The Effect of Conjugated Linoleic Acid (CLA) Isomers on Hepatic Lipid Droplets and Lipid Droplet Proteins in *fa/fa* Zucker Rats

by

Nazanin Kazem Moosavi

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

Master of Science

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
*****
COPYRIGHT PERMISSION

The Effect of Conjugated Linoleic Acid (CLA) Isomers on Hepatic Lipid Droplets and Lipid Droplet Proteins in *fa/fa* Zucker Rats

By

Nazanin Kazem Moosavi

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

Master of Science

Nazanin Kazem Moosavi©2009

ABSTRACT

Type 2 diabetes and nonalcoholic fatty liver disease (NAFLD) are some of the consequences of obesity. It is hypothesized that conjugated linoleic acid (CLA) can be used as a therapeutic agent to treat NAFLD in *fa/fa* Zucker rats through its ability to reduce lipid droplet formation and decrease the cellular level of associated lipid droplet proteins (adipophilin and perilipin).

The first objective was to determine the effects of CLA isomers (0.4% *cis-9,trans-11* and 0.4% *trans-10,cis-12*) on the number and size of lipid droplets and the lipid droplet proteins adipophilin and perilipin in 17 week old *fa/fa* Zucker rats after an 8 week dietary intervention. The second objective was to determine the effects of peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR) and farnesoid X receptor (FXR) agonists on adipophilin and perilipin in a cell culture model of lipid accumulation using hepatic H4IIE cells.

Treatment with the *cis-9,trans-11* and *trans-10,cis-12* CLA isomers reduced the lipid droplet area compared to control group but the number of lipid droplets was similar in all *fa/fa* rats regardless of dietary treatment. The *trans-10,cis-12* CLA isomer significantly decreased hepatic adipophilin but not perilipin protein levels in the *fa/fa* rats. The cell culture study showed that cells treated with the PPARγ agonist rosiglitazone and the PPARα agonist WY14643 had lower amounts of adipophilin protein compared to the other treatments.

In conclusion, the anti-steatotic effects of *trans-10,cis-12* CLA in older *fa/fa* Zucker rats with established obesity and metabolic syndrome were associated with

reduced levels of the lipid droplet protein adipophilin as well as smaller lipid droplet area in the liver. The cell culture study indicated that adipophilin may be regulated by PPARα and PPARγ, and reductions in lipid accumulation and adipophilin levels in H4IIE cells are mediated by PPARγ and PPARα, but not LXR or FXR.

ACKNOWLEDGEMENTS

DEDICATION

In memory of my father Mr. Seyedaliasghar Kazem Moosavi, the man who inspired me
all of my life and my mother Azarmidokht Nourainejad.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

9-11	*cis*-9,*trans*-11 CLA diet
10-12	*trans*-10, *cis*-12 CLA diet
ACO	acyl-CoA oxidase
ALT	alanine aminotransferase
ANOVA	analysis of variance
BA	bile acid
BMI	body mass index
BSA	bovine serum albumin
MEM Alpha	Minimum Essential Medium Eagle, Alpha
c9,t11	*cis*-9, *trans*-11
CIC	citrate carrier
CLA	conjugated linoleic acid
CTL	control diet
DBD	DNA-binding domain
DM-2	diabetes mellitus-type 2
ddH$_2$O	distilled, deionized water
kDa	kilodalton
MUFA	monounsaturated fatty acid
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
LXR	liver-X-activated receptor
LBD	ligand binding domain
OA	oleic acid

ob/ob	obesity gene
OCT	optimal cutting temperature compound
OLETF	Otsuka Long-Evans Tokushima fatty
PBS	phosphate-buffered saline
PO	pioglitazone
PPAR	peroxisome proliferators – activated receptors
PVDF	polyvinylidene
RE	response element
RO	rosiglitazone
RXR	retinoic acid receptor
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SCD-1	stearoyl-CoA desaturase-1
SEM	standard error of mean
SFA	saturated fatty acid
SREBP	sterol regulator element-binding protein
TAG	triacylglycerol
TBS	Tris buffered saline
TBST	Tris-buffered saline with Tween-20
TEMED	N, N, N', N'-Tetramethylethylenediamin
TNF-α	tumor necrosis factor-α
TZD	thiazolidinediones
t10,c12	*trans*-10, *cis* -12
VLDL	very-low density lipoprotein

WHO	World Health Organization
wt/wt	weight by weight
ZDF	Zucker diabetic fatty

The effect of conjugated linoleic acid (CLA) isomers on hepatic lipid droplets and lipid droplet proteins in *fa/fa* Zucker rats

LITERATURE REVIEW

Excessive body weight is one of the most important risk factors for various diseases worldwide. Cardiovascular disease, type 2 diabetes, cancer, liver disease and other complications are some of the consequences of obesity. Today, there is a growing interest in finding molecules including dietary components that have beneficial effects on obesity and its complications. The purpose of the following literature review is to investigate current knowledge about conjugated linoleic acid (CLA) as a potential therapeutic agent for the management of certain characteristics of metabolic syndrome, especially obesity and non-alcoholic fatty liver disease (NAFLD). CLA is a mixture of positional and geometric isomers of linoleic acid, and it has attracted considerable attention because of its potentially beneficial biological effects both *in vitro* and *in vivo*. NAFLD is emerging as an important complication of obesity.

Nonalcoholic Fatty Liver Disease (NAFLD)

Nonalcoholic fatty liver disease (NAFLD) refers to a wide range of liver diseases including simple fatty liver (steatosis), nonalcoholic steatohepatitis (NASH), cirrhosis, and end-stage liver disease (Angula, 2005). It is emerging as the most common liver disease in industrialized countries. Confirmation of the disease can be achieved by imaging studies and staging the disease requires a liver biopsy. Treatment is based on

weight loss and exercise, although insulin-sensitizing agents, antioxidants and medications are promising (Papandreou, 2007). The risk of NASH progressing to cirrhosis is approximately 20% in 10-20 years (Younossi et al., 2002). NAFLD is one of the most prevalent forms of the liver diseases. The prevalence of NAFLD is 20% and the prevalence of NASH is 2-3% in general population in the United States (Younossi et al., 2002).

Hepatic steatosis is the accumulation of triacylglycerols (TAG) in the liver cell and is diagnosed when lipid content in the liver exceeds 5-10% by weight (Reddy et al., 2006). This could be accompanied by clinical signs such as insulin resistance and liver malfunction. Numerous conditions contribute to NAFLD, but the exact etiology is still unknown. Obesity and insulin resistance are associated with the increased prevalence of hepatic steatosis. Hepatic steatosis is a common feature of metabolic syndrome. Currently 75% of persons with obesity and diabetes have some degree of NAFLD (Purushotham et al., 2007). The proportion of obese children tripled in last 25 years, and currently, two out of three adults in Canada are overweight or obese (Health Canada, 2009a).

Type 2 Diabetes (DM-2)

Type 2 diabetes (DM-2) is an increasingly common cause of morbidity and mortality in the world. It is reported to affect 3-5% of the population worldwide and 10-60% of individuals over 60 years of age (Leonard et al., 2005). In 2005-2006, Canada had approximately 2 million people diagnosed with diabetes; this represents about 1 in 17 Canadians. The number of people with diagnosed diabetes continues to grow. Two

hundred thousand new cases of diabetes were diagnosed in 2005-2006; however, it is now recognized that a large number of individuals are not aware they have this disease (Public Health Agency of Canada, 2009).

DM-2 occurs when target cells cannot take up glucose in sufficient amounts and blood glucose become elevated. It is characterized by a reduced sensitivity to the effects of insulin and eventually results in impaired β-cell function (Upton et al., 1998). Many people with DM-2 also have metabolic syndrome. There is a decreased ability of insulin to stimulate the use and storage of glucose which results in hyperglycemia and diabetes.

Obesity is one of the major risk factors for DM-2. Obesity is defined as a body mass index (BMI) of greater than 30 kg/m^2 (Health Canada, 2009b). BMI is a simple index of weight-for-height that is commonly used in classifying overweight and obesity in adult populations and individuals. It is defined as the weight in kilograms divided by the square of the height in meters. Obesity and DM-2 are also risk factors for NAFLD. In obese animals, there is an increase in glucose conversion to lipid in all tissues, including the liver, due to an increase in lipogenesis. Insulin-stimulated glucose uptake is also significantly less in skeletal muscle (Leonard et al., 2005).

Insulin resistance in DM-2 may be due to low numbers or lower activity of insulin receptors or loss of post receptor function (Sugden et al., 2008). The Zucker Diabetic Fatty (ZDF) rat is a commonly used animal model of DM-2. These rats were derived from the inbreeding of hyperglycemic Zucker obese rats. ZDF rats have a mutation in the extracellular domain of the leptin receptor. This mutation impairs the appetite suppressing effects of leptin and it also has thermogenic effects. These animals exhibit

obesity, hyperphagia, polyuria, polydipsia, and have insulin resistance. Hyperglycemia develops when these animals are between 7-12 weeks (Leonard et al., 2005).

Metabolic Syndrome

NAFLD is associated with dyslipidemia, obesity, and insulin resistance, which are the main features of metabolic syndrome. Metabolic syndrome is a major public health concern. Metabolic syndrome is a cluster of characteristics including abdominal obesity, atherogenic dyslipidemia, raised blood pressure, insulin resistance, a prothrombotic state, and a pro-inflammatory state (National Cholesterol Education Program, 2009). Overweight/obesity, physical inactivity and genetic factors are risk factors for the metabolic syndrome.

The therapeutic options for managing individuals with metabolic syndrome usually target the major symptoms and are designed to reduce body weight and hypertension, and improve lipid and glucose irregularities. The beneficial effects of CLA, such as body fat reduction in mice and liver lipid lowering in rats, have led to suggestions that CLA could be used for the treatment of the metabolic syndrome.

The *fa/fa* Zucker rat is a good model of obesity and the metabolic syndrome. The *fa/fa* Zucker rat develops metabolic and hormonal disorders that share many features with human obesity. The *fa/fa* Zucker rats have hyperphagia due to a missense mutation on the leptin receptor gene; they become obese, hyperinsulinemic, hypertensive and have NAFLD (Taylor and Zahradka, 2004).

Conjugated Linoleic Acid (CLA)

Conjugated linoleic acid (CLA) refers to a mixture of naturally occurring positional and geometric isomers of conjugated dienoic derivatives of linoleic acid (Wang et al., 2004). CLA is produced by bacteria via biohydrogenation of linoleic and α-linoleic acid in the ruminant intestine (Granlund et al., 2003; Ferramosca et al, 2006). The *cis*-9, *trans*-11 (c9,t11) CLA isomer is the major isomer in ruminant meats (beef, dairy, sheep, and goat), ruminant milk, dairy products (yogurt, cheese, butter) and human breast milk. The CLA content of cheese ranges between 3.59 to 7.96 mg CLA/g lipid (Lin et al., 1995). Fermented dairy products contained 3.82 to 4.66 mg CLA/g lipid. Fluid milk contains 3.38-6.39 mg CLA/g lipid and its CLA content changes based on seasonal and geographical situation (Banni, et al., 1996). CLA content in cow's milk is higher in spring and summer due to the presence of more polyunsaturated fatty acids in their diet (Kraft et al., 2003). CLA in milk or meat is stable during cooking and storing. Human CLA intake is varies between 151 mg for women and 212 mg for men for those not consuming CLA commercial preparations, however, these amounts are variable based on the individual's diet.

The c9,t11 isomer accounts for more than 90% of the total CLA intake from dietary sources (Silvereira et al., 2007). Many feeding studies use synthetically produced CLA which contains a mixture of c9,t11 and t10,c12 isomers. These isomers of linoleic acid have been shown to have anticarcinogenic, antiatherogenic, antidiabetes, and antiobesity properties (Granlund et al., 2003). The rapid increase in the prevalence of obesity has attracted considerable attention to CLA because of its beneficial effects to

5

decrease body fat mass while enhancing lean body mass in some species (Tsuboyama-Kasaoka et al., 2000).

CLA is able to reduce adiposity by affecting energy and lipid metabolism (Leaver et al., 2006). CLA reduces body fat mass in mice, rats, hamsters and pigs; however, CLA causes liver enlargement and increases tissue lipid content in mice (Takahashi et al., 2003). Results in humans have been inconsistent. In overweight/obese subjects, 4 of 7 studies observed a reduction in body fat whereas the others reported no change (Lee et al., 1994). The different results with respect to species and age-related responses may be due to factors such as the treatment dose, duration and background diet.

Studies have demonstrated that the c10,t12 isomer of CLA is most likely responsible for reducing body fat (Ferramosca et al., 2006). CLA administration in mice decreases body fat mass, but it produces lipid accumulation in the liver. Although CLA reduces body fat, the enlargement of the liver has raised concerns about its safety. In C57BL/6J mice, dietary supplementation with 1% c9,t11 and t10,c12 CLA for 1 week caused massive fatty liver (Wang et al., 2004). Wang et al., (2005) investigated the effects of short-term feeding with 2% CLA for 1 week on adipose tissue weights, liver weight and hepatic lipid metabolism in C57BL/6J mice. In this study, short-term feeding of CLA resulted in lipodystrophy in C57BL/6J mice without inducing adverse effects in the liver.

Other researchers have investigated effects of CLA on hepatic steatosis and adiposity in rat models. Purushothem et al., (2007) investigated the effect of a CLA mixture (39.2% c9,t11 and 38.5% t10,c12) on liver lipid in a rodent model for hepatic steatosis which is resistant to the adipose-lowering effects of CLA. Obesity in these rats

was induced by diet and without any genetic manipulation. After feeding the Wistar rats with a high fat diet (20% fat) for four weeks to induce obesity and hepatic steatosis, the diet was switched to a low fat diet (6.5% fat) with either soybean oil or soybean oil and the CLA mixture for an additional four weeks. The rats fed CLA during the low fat phase had significantly decreased hepatic lipid accumulation without altering adipose mass. CLA significantly increased the mRNA level of PPAR-α and increased peroxisomal oxidation in the liver (Purushotham et al., 2007).

Nagao et al. (2005) tested whether dietary CLA protects 6 week old male *fa/fa* Zucker rats from hepatic injury. The CLA group was fed a 1% CLA mixture (46% c9,t11, 47.3% t10,c12) and compared with a control group in which CLA was replaced with 1% linoleic acid. After 8 weeks of feeding, hepatomegaly and hepatic TAG accumulation were reduced in the CLA-fed Zucker rats compared with the control group. It was suggested that hepatic steatosis resulted from increased hepatic β-oxidation. In the CLA diet group, the activity of carnitine palmitoyltransferase (the key enzyme of fatty acid β-oxidation) was enhanced and microsomal TAG transfer protein (a factor for lipoprotein secretion) was improved. They speculated that an elevation in plasma adiponectin and a reduction of liver tumor necrosis factor-α (TNF-α) mRNA in the CLA-fed *fa/fa* Zucker rats may be associated with the improvement in hepatic steatosis. CLA acts as an inducer of adiponectin and adiponectin is primarily secreted from adipose tissue in rodent and human. Adiponectin has been reported to alleviate alcohol or obesity-induced hepatomegaly, and hepatic steatosis in mice (Xu et al., 2003). In humans, adiponectin has a protective role against NAFLD (Bajaj et al., 2004; Nagao et al., 2005).

A study by Wendel and Belury (2006) investigated the effects of 2 week diet supplementation with either 1.5% CLA or 0.2% troglitazone (TZD) in 6 week old male ZDF rats. Thiazolidenediones such as troglitazone are insulin sensitizing agents that can lower fasting blood glucose and reduce hepatic TAG levels and are used in DM-2 therapy. Thiazolidenediones function as high affinity ligands for nuclear receptor peroxisome proliferator activated receptor-gamma (PPARγ). PPARγ stimulates the differentiation, proliferation, and lipid accumulation of adipocytes, and promotes lipid accumulation in adipose tissue, but prevents lipid accumulation in peripheral tissues such as liver. The study by Wendel and Belury (2006) compared CLA and the thiazolidenedione troglitazone because many of the antidiabetic effects of CLA and thiazolidinediones are similar. Lipid accumulation and body composition of both lean and ZDF rats were measured after 2 weeks of feeding. ZDF with control diet developed significant hepatic steatosis while the hepatic TAG level in both CLA-fed and TZD-fed ZDF rats were similar to those of lean rats. Also, the ratio of 16:1/16:0 and 18:1/18:0 fatty acids were reduced in the liver of ZDF rats fed either the CLA or TZD diet. The 16:1/16:0 and 18:1/18:0 ratio is a marker for stearoyl-CoA desaturase-1 (SCD-1) activity. The CLA diet reduced adipose mass, however, TZD had no effect. The results showed that both TZD and CLA improved hepatic steatosis and fatty acid composition in ZDF rats, and suggests that changes in hepatic lipid composition maybe associated with a reduction in SCD-1.

Mechanism(s) of Action for CLA in Hepatic Steatosis

In an effort to understand the mechanism by which CLA modulates hepatic steatosis, different hypotheses have been suggested. One of the suggestions is that CLA affects the activity of the nuclear receptor family of transcription factors, which includes PPARα, PPARγ, liver X receptor-alpha (LXRα) and Farnesoid X receptors (FXR) (Taylor and Zahradka, 2004). These transcription factors will be discussed in more detail in another section.

CLA incorporation into cellular lipid alters fatty acid composition and can inhibit lipogenesis and TAG esterification by disrupting the fatty acid desaturation process. SCD-1 also known Δ9 desaturase is one of key desaturation enzymes in lipogenesis. Desaturation of saturated fatty acids (SFA) is necessary to produce TAGs. It has been suggested that CLA reduces SCD-1 mRNA expression and activity, and inhibits *de novo* fatty acid and TAG synthesis (Choi et al., 2000). This is supported by observations that CLA treatment increases the saturated (SFA):monounsaturated fatty acid (MUFA) ratio (Purushotham et al., 2007).

Other hypotheses focus on changes in energy metabolism due to CLA (Sakona et al., 1999). In mice, CLA increases energy expenditure, fatty acid oxidation and lipolysis, and this could alter the amount of lipid in the liver. The effects of CLA on energy expenditure and lipid metabolism depend on isomer type, dose and duration of the diet as well as the metabolic status and species of the experimental subjects. The t10, c12 CLA isomer is more efficiently oxidized than other isomers (Evans et al., 2002). In mice, it has been demonstrated that CLA increases the activity of important enzymes associated with hepatic lipogenesis. Hepatic lipogenesis occurs partly in the mitochondrial matrix

and partly in the cytosol. These two different compartments are connected with a mitochondrial transport protein called citrate carrier (CIC) which has an important role in intermediary metabolism. The CIC activity is down-regulated during starvation, while in mice fed diet enriched with a mixture of c9,t11 and t10,c12 for 16 weeks, CIC activity was increased along with an elevation of hepatic TAGs (Ferramosca et al., 2006).

Our laboratory has begun to study the effects of CLA on lipid droplet proteins as a new mechanism for understanding effects of CLA on hepatic steatosis (Stringer et al., 2009). In vertebrate animals, most of the energy is stored in the lipid droplets, primarily in adipocytes. Other tissues such as liver also have lipid droplets. The next section will describe the proteins that control lipid droplet formation and hydrolysis, lipid traffic in cells and the regulation of whole body energy metabolism.

Lipid Droplets and Lipid Droplet Proteins

Adipocytes hold the body energy reserves as TAG in lipid droplets. Lipid droplets can be as large as 100 μm and they have a hydrophobic TAG core, a phospholipid and cholesterol monolayer and embedded proteins (Brasaemle et al., 2004). Obesity increases TAG storage in adipose depots. When this TAG storage overflows, free fatty acids are released and accumulate as TAGs in non-adipose tissues, a process that is called steatosis (Wolins et al., 2006). These non-adipose tissues are not adapted for excess TAG storage and this causes complications such as dyslipidemia, insulin resistance, β-cell failure and hypertension.

The majority of TAG in mammalian cells is in droplets coated with one or more members of the perilipin-adipophilin-TIP47 (PAT) family (Bickel et al., 2009). Perilipin

and adipophilin are the most abundant proteins on these lipid droplets. The ideal location for perilipin and adipophilin to able to regulate the lipid pool is on the surface of lipid droplets. These proteins play a critical role in both TAG synthesis and hydrolysis. Mice lacking perilipin cannot accumulate large adipose TAG stores and hormone regulated lipolysis is muted (Wolins et al., 2006). This shows that perilipin inhibits lipolysis and promotes TAG storage in fed animals. Perilipin is largely limited to adipose tissue and steroidgenic cells, whereas adipophilin (adipose differentiation related protein, ADRP) has a broad tissue distribution. The three protein isoforms of perilipin are named perilipin A, B and C and they are the result of alternative splicing of a single gene transcript (Brasamle et al., 2004).

The mechanism of how perilipin controls TAG stores is still under investigation. Adipophilin in non-adipocytes inhibits TAG hydrolysis, the same role as perilipin in adipocytes. In cultured cells (murine fibroblast cell lines, 3T3-L1, NIH-3T3 and Swiss-3T3), the over-expression of perilipin or adipophilin increases TAG storage in the cell (Wolins et al., 2006 & Imamura, et al., 2002). Adipophilin is less protective against TAG hydrolysis than perilipin (Wilson et al., 2006). The stimulation of lipolysis by β-adrenergic agonists triggers phosphorylation of perilipin and causes translocation of hormone sensitive lipase to the surface of lipid droplets resulting in gradual fragmentation and dispersion of lipid droplets.

Adipophilin and perilipin are proteins which associate with the lipid droplets of adipocytes and their function is to control lipolysis. Perilipin protects TAGs from lipases and helps to promote TAG storage. The mechanism by which perilipin controls adipocyte lipolysis is based on the nutritional status. In the fed state, phosphorylation of perilipin

leads to the formation of a barrier at the surface of the lipid droplet which restricts the access of lipase to TAG. This effect reduces the rate of catalysis. Perilipin also acts as a scaffold at the surface of lipid droplets and in this way acts as an organizer for enzyme metabolism. In the fed state, proteins bind to this scaffold, stabilize the lipid droplets and protect them from lipolysis. Upon stimulation of lipolysis, lipid droplet remodeling is stimulated and this promotes motility of micro droplets (Brasaemle et al., 2008).

CLA, Lipid Droplet Proteins, and Hepatic Steatosis

It is possible that agents with the ability to reduce lipid droplet protein expression could inhibit hepatic steatosis. Our laboratory has reported that dietary CLA supplementation can reduce the hepatic accumulation of lipids in *fa/fa* Zucker rats (Noto et al., 2006). More recently, Stringer et al. (2009) examined the effects of CLA isomers on lipid droplet proteins in this model. Six-week old male *fa/fa* Zucker rats were fed four different diets (0% CLA, 0.4% c9,t11 CLA, 0.4% t10,c12 CLA, and a mixture of 0.4% c9,t11 CLA and 0.4% t10,c12 CLA) for 8 weeks. The results of this study showed that liver lipid concentration decreased in the t10,c12 CLA and CLA mixture groups, and this was associated with improvemed liver function as determined by alanine aminotransferase (ALT). Liver adipophilin levels were significantly lower in the t10,c12 group compared to the control group, but there were no changes observed in perilipin among the groups (Stringer et al., 2009). To my knowledge, this is the first study to show the effect of CLA on lipid droplet proteins in a model of hepatic steatosis.

The *fa/fa* Zucker Rat as a Model for Hepatic Steatosis

There are several genetic rodent models for obesity, metabolic syndrome and diabetes. In mice, the obesity and diabetes syndromes are caused by two single autosomal recessive mutations, obese (*ob*) and diabetes (*db*). The *ob/ob* mice are unable to produce satiety factor (leptin), while *db/db* mice are resistant to it due to a mutation in the leptin receptor. The *ob* gene encodes leptin which is expressed specifically in the adipose tissue while the leptin receptor is expressed in several tissues. Leptin acts as a sensor of fat mass.

The rat gene for fatty (*fa*) is a homologue of the mouse *db* gene, and *fa/fa* rats have a mutation in the extracellular domain of the leptin receptor (Takaya etal.1996). The *fa/fa* rats accumulate adipose mass soon after birth and obesity becomes apparent as early as 3 weeks of age (Truet et al., 2000). The Zucker rats develop a syndrome that shares many features with human obesity such as hyperphagia, hyperinsulinemia (by 4-5 weeks), hypertension (at six weeks) and NAFLD (Nagao et al., 2005). Insulin and leptin resistance develop in parallel and both could contribute to hepatic lipid accumulation. However, a study by Fishman et al. (2007) provides evidence that failure of leptin action is the primary determinant for hepatic steatosis. Thus, in the fa/fa Zucker rat, hepatic steatosis could be due to leptin resistance since leptin plays an important role in regulating fat metabolism and fat distribution. Unlike ZDF rats, they do not develop hyperglycemia and diabetes. In *fa/fa* rats, hyperinsulinemia is present and indicates an increase insulin levels in the blood due to insulin resistance. The *fa/fa* Zucker rat is considered a model of the prediabetic state with hepatic steatosis (Nagao et al., 2003a).

Role of Transcription Factors and Nuclear Receptors in Lipid Metabolism

The effects of CLA can be explained in part by its relationship to nuclear receptors. The nuclear receptor super family describes a family of related but diverse transcription factors. This family includes the nuclear hormone receptors (NHRs) and various orphan nuclear receptors (Sugden et al., 2008). The difference between NHRs and orphan receptors is based on their ligands. The NHRs have known ligands but orphan receptors do not. These receptors are located in the cell interior and lipophilic hormones traverse the plasma membrane and bind to specific receptor proteins in the cytosol, which then transduce the signals to the nucleus. Although the structure and function of NHRs are different, they have a variety of common features. NHRs have a ligand binding domain (LBD) which controls receptor activation, a process that is dependent on the presence of bound ligands. These receptors also have a central DNA-binding domain (DBD). For example, activation occurs when PPAR forms a heterodimer partnership with the retinoid X receptor (RXR). After activation, the hormone-receptor complexes migrate to the nucleus, and bind to specific DNA sequences called response elements (REs). These elements regulate the expression of genes for various metabolic processes. For example, PPAR response elements (PPRE) regulate lipid metabolism (Jerrold et al., 2001; Novack et al., 2004).

Recently, interest has developed in the utility of nuclear hormone receptors for the treatment of diabetes and lipid abnormalities (Wanger et al., 2008). To develop hepatic steatosis, TAG must accumulate in hepatocytes. This accumulation may occur for different reasons, such as an excess of dietary lipid, increased TAG synthesis, diminished

export of TAG or reduced fatty acid oxidation (Wierzbicki et al., 2009). Nuclear receptors play an important role in lipid metabolism, particularly PPARs, LXR, and FXR (Gaemers et al., 2006) and thus it has been proposed that nuclear receptor ligands could be used to modulate hepatic lipid metabolism and hepatic steatosis. The following section will described PPARs, LXR and FXR in more detail.

Peroxisome proliferator – activated receptors (PPARs)

There are three isoforms of PPAR: PPARα, PPARδ (also known as PPARβ) and PPARγ. Each PPAR has its own pattern of expression and biological activities. In the body, the distribution of each isoforms is unique. Likewise, each has a distinct role in modulating lipid metabolism (Chinetti et al., 2009; Adidaet al., 2002).

Peroxisome proliferator-activated receptor alpha (PPARα)

PPARα is prominently expressed in tissues that have a high rate of lipid catabolism, such as liver, kidney, heart and skeletal muscle (Chinetti et al., 2009). Eicosanoids, free fatty acids, and fibrate drugs can activate PPARα. This activation causes an increased uptake and oxidation of free fatty acids, and increased TAG hydrolysis (George et al., 2008). PPARα induces the expression of genes for enzymes involved in fatty acid β- oxidation and ω-oxidation, and it also affects proteins that are involved in fatty acid uptake and transport (Adida et al., 2002). For example, PPARα regulates acyl CoA oxidase (ACO), the rate limiting enzyme in the β-oxidation of long chain fatty acids (George et al., 2008). Fibrate treatment induces fatty acid transport protein expression in rat liver and increases acyl-CoA synthetase mRNA levels (Martin et

15

al., 1997). Studies have shown that the PPARα agonist Wy14643 plays an important role in fatty acid disposal pathways that affect the pathogenesis of hepatic steatosis (Gearge et al., 2008).

Peroxisome proliferator-activated receptor delta (PPAR δ)

In the human body, PPARδ is the most widespread PPAR isotype (Bilityet al., 2008). It is highly expressed in liver and intestine, and found in a wide variety of tissues including adipose and muscle (Chinetti et al., 2009). PPAR δ has a key role in reverse cholesterol transport and it also affects adipogenesis (Taylor et al., 2004). PPARδ has become of interest for the treatment of metabolic syndrome and cardiovascular disease. It has been shown that PPARδ increases oxidative metabolism (fatty acid oxidation) and reduces adipose tissue mass. Furthermore, PPARδ activation mimics the effects of physical exercise (Wanger et al., 2008). PPARδ is activated by synthetic agonists such as GW0742. There is strong evidence that agonists which activate PPARδ could be used as therapeutic agents for diabetes, dyslipidemias and metabolic syndrome (Bilityet al., 2008).

Peroxisome proliferators-activated receptor gamma (PPARγ)

PPARγ is highly expressed in adipose tissue but is also found in the vascular endothelium, pancreatic β-cells, hepatocytes, hepatic stellate cells and macrophages (George, 2008). PPARγ is a metabolic regulator, which affects adipose tissue, skeletal muscle, liver and heart. PPARγ activation increases the transport and oxidation of fatty acids, and improves glucose homeostasis due to inhibition of hepatic glucose output (George et al., 2008).

16

Two different kinds of PPARγ ligands have been identified: natural and synthetic. The natural PPARγ ligands include long chain polyunsaturated fatty acids and eicosanoids, while the synthetic PPARγ ligands include the thiazolidinedione drugs (TZDs) (Zhou, 2007). Activating PPARγ with prostaglandins or drugs causes terminal differentiation and proliferation of subcutaneous adipocytes. This is due to an increase in fatty acid uptake by adipocytes and it prevents lipid overloading of non-adipose tissues such as liver. PPARγ activation by TZDs also improves insulin sensitivity. For these reasons, TZDs are widely used as drugs for the treatment of DM-2. Different compounds belong to this drug class, and two synthetic PPARγ agonists, pioglitazone and rosiglitazone, are approved by FDA for treatment of DM-2 (Milglio et al., 2009). PPARγ activators are also used to treat other components of the metabolic syndrome such as obesity, dyslipidemia, hyperglycemia, insulin resistance and may be used for nonalcoholic steatohepatitis (George et al., 2008).

Liver X Receptor (LXR)

LXRs, including the α and β isoforms, are ligand-activated transcription factors that belong to the nuclear receptor super family. The expression of LXRα is abundant in the liver, spleen, kidney, adipose and small intestine, whereas LXRβ is expressed in almost all tissues (Yoshikawa et al., 2002). Like other nuclear receptors, LXR is an obligated partner with retinoid X receptors (RXR). They have a key role in lipid metabolism and inflammation, and therefore, LXR agonists can be used for the treatment of different diseases such as DM-2. LXRs are activated by oxysterols or by the synthetic

LXR agonist GW3965. GW3965 significantly induces the expression of LXR lipogenic target genes (Sholz et al., 2009).

Farnesoid X Receptor (FXR)

FXR is expressed in liver, gall bladder, intestine, kidney and adrenal gland (Bass et al., 2009). To modulate target gene transcription, FXR is required to heterodimerize with RXR (Martinez-Fernantez et al., 2009). FXR is involved in sterol metabolism and lipogenic pathways (Sugden et al., 2008). A key function of FXR is hepatoprotection, and this is achieved by controlling the level of bile acids (Wanger et al., 2008). FXR coordinates the expression of genes involved in the conversion of cholesterol to bile acids (Feng et al., 2009). Besides the critical role of FXR in bile acid metabolism, FXR regulates glucose metabolism. FXR activity is stimulated by bile acids or synthetic agonists such as GW4064 (Cariou et al., 2005). GW4064 is a non-steroidal FXR agonist which can reduce hyperlipidemia in some animal models (Feng et al., 2009).

New studies have suggested FXR may have much broader physiological and pathological functions in the liver (Wanger et al., 2008). It has broad activities in non-metabolic functions such as liver regeneration (Kaimal et al., 2009). Specially, FXR protects the liver by preventing the toxic effect of bile acids when they reach high levels. FXR null mice develop liver tumours due to liver injury.

H4IIE Cells as the Cell Culture Model

H4IIE hepatoma cells originate from liver tissue. In early studies of hepatic cells, the metabolic flexibility of hepatic cells and the effect of dietary intake and enzyme

content of the liver were identified (Knox et al., 1956). A hepatoma is the neoplastic hepatic cell and has been mainly studied in relation to enzymatic differences with normal hepatic cells. Interest in the mammalian hepatic cells began when the cell function governing the relationship between the cell and external environment was recognized (Pitot et al., 1964). The H4IIE line was established in 1961 from the Reuber H35 hepatoma (Evan et al., 1977). H4IIE cells are primarily used for the study the signaling molecules associated with cell proliferation due to distinct responses to early signaling events triggered by the ligands. H4IIE hepatoma cells are considered a poorly differentiated hepatoma, with loss of connective tissue and increasing in growth rate. Accelerated growth of H4IIE cells is result of: (1) having a shorter cell cycle time, (2) increasing numbers of proliferating cells and (3) profound decreases in cell losses (Evan et al., 1977). These characteristics of H4IIE cells make them suitable for studies of cell growth and metabolic process. The H4IIE cell line can be maintained either in cell culture or transplantable solid tumour (Evan et al., 1977). In the quiescent state, cells are healthy and viable, and have the potential to initiate the cascade of events required for enhanced growth. The quiescent state (steady – state level of DNA synthesis) is obtained by incubating the cells in serum free media or 72 h (Lau et al., 1998). For this thesis, H4IIE cells were chosen as a convenient model to investigate the effects of PPAR, LXR and FXR agonists on adipophilin and perilipin for prevention of lipid droplet formation.

STUDY RATIONALE

Lipid droplets and the proteins that coat them is a new emerging area of study that is expected to improve our understanding of the underlying mechanisms that lead to diseases of lipid accumulation. Damage to liver function and failure to package fatty acids appropriately could be associated with NAFLD in diabetes and obesity. Adipophilin and perilipin have an important role in controlling fatty acid flux. Thus, the aim of this thesis was to determine if these proteins are affected by CLA treatment and whether a pharmacological dose of CLA could promote lipid packaging and thereby limit lipotoxicity in vulnerable tissues such as the liver.

Previously, our laboratory has demonstrated that supplementation with t10,c12 CLA for 8 weeks reduces hepatic steatosis and the volume of lipid droplets in growing male *fa/fa* Zucker rats (Stringer et al., 2009). The t10,c12 CLA treatment decreased hepatic adipophilin but did not change perilipin when 6 week old *fa/fa* Zucker rats were supplemented with CLA for 8 weeks. The animal study in this thesis investigates the effect of CLA isomers on lipid droplet proteins in the liver of older *fa/fa* Zucker rats with established disease (17 week old *fa/fa* rats supplemented with CLA isomers for 8 weeks). Hepatic steatosis has been assessed in these older Zucker rats and the liver lipid in the *fa/fa* rats fed t10,c12 CLA was reduced to levels similar to the lean rats fed control diet (Taylor and Zahradka, unpublished data). Thus, the goal of this thesis was to determine the effects of CLA isomers on lipid droplets and lipid droplet proteins in relation to CLA effects on hepatic steatosis in older *fa/fa* Zucker rats.

The cell culture experiment in this thesis was to determine whether the results of the *in vivo* studies are supported by experiments using an *in vitro* model of liver lipid

20

accumulation. H4IIE cells were used to examine if adipophilin and perilipin proteins are affected by different ligands of PPARs, FXR and LXR (*in vitro*). We also investigated if a pharmacological dose of the PPAR, FXR and LXR ligands could promote lipid packaging.

Hypotheses

It was hypothesized that:

1. The t10,c12 CLA diet improves hepatic steatosis in *fa/fa* Zucker rats by reducing lipid droplet formation and decreasing the associated lipid droplet proteins (adipophilin and perilipin).

2. The c9,t11 CLA diet does not affect hepatic steatosis in this model and thus lipid droplets and lipid droplet proteins will not be altered compared to *fa/fa* rats fed the control diet.

3. PPAR ligands will affect lipid droplet accumulation and lipid droplet proteins in a cell culture model of lipid accumulation.

Objectives

To investigate the above hypotheses, the following objectives were defined:

1. To determine the effects of 8 week dietary intervention with CLA isomers in 17 week old *fa/fa* Zucker rats on the number and size of lipid droplets in liver (by histochemistry and image analyses).

2. To investigate the effects of CLA isomers on the lipid droplet proteins, adipophilin and perilipin (by Western blotting), in liver from *fa/fa* Zucker rats.

3. To determine the effects of PPAR, LXR and FXR ligands on lipid accumulation (by visual inspection), and adipophilin and perilipin proteins (by Western blotting) in a cell culture model of lipid accumulation using hepatic H4IIE cells, given that CLA can alter the nuclear receptor activity.

RESEARCH DESIGN & METHODS

Animal Study

Animals and Dietary Treatments

The liver tissue analyzed in this thesis was acquired from a previously completed animal study. For that study, 16 week old male lean (ln) and *fa/fa* (fa) Zucker rats (Harlan, Indianapolis, IN) were housed individually in stainless steel hanging cages. After being acclimatized to their new environment for five to eight days, the rats were randomly assigned to one of five groups (n=10 per group): *fa/fa* baseline (fa Base), *fa/fa* fed control diet (fa CTL), *fa/fa* fed 0.4% (w/w) c9,t11 CLA (fa 9-11), *fa/fa* fed 0.4% (w/w) t10,c12 CLA (fa 10-12), and lean rats fed control diet (ln CTL). The fa Base group was euthanized for tissue collection as described below. The remaining rats were maintained on their experimental diet for 8 weeks and fed *ad libitum* except for an additional group of *fa/fa* rats that were in the pair-weighed group (feed intake restricted so that this group weighed the same as the CLA group with the lowest body weight). This pair-weighed group was included to determine whether differences in measured parameters in the CLA groups were due to CLA and not due to changes in body weight. However, the CLA groups weighed the same as the fa CTL group throughout the study; thus, the feed intake of the pair-weighed group was not restricted and samples from this group were not analyzed.

The composition of the diets is shown in Table 1. The diets were freshly prepared each week and stored frozen at -20°C until used. Rats were given new feed cups with

Table 1: Diet Formulation

Ingredient (g/kg) [1]	c9,t11	t10,c12	CTL
Cornstarch [2]	363	363	363
Maltodextrin	132	132	132
Sucrose	100	100	100
Egg white	212.5	212.5	212.5
Cellulose	50	50	50
Mineral mix, AIN-93G-MX	35	35	35
Vitamin mix, AIN-93-VX	10	10	10
Choline	2.5	2.5	2.5
Biotin Mix [3]	10	10	10
Tert-butyl hydroquinone [4]	0.014	0.014	0.014
Soybean oil	80.6	80.6	85
c9,t11 CLA [5]	4.4	--	--
t10,c12 CLA [5]	--	4.4	--

[1] Ingredients purchased from Harlan Teklad (Madison, WI) unless otherwise indicated.

[2] Castco Inc. (Etobicoke, ON)

[3] 200 mg biotin/kg cornstarch because egg white was the protein source

[4] Sigma-Aldrich Chemical Co. (St. Louis, MO)

[5] Larodan Fine Chemicals (Malmö, Sweden)

fresh feed three times per week. Feed intake was corrected for spillage. Body weight was recorded every week.

Tissue Collection

At the end of the study period, after a 12 h overnight fast, rats were euthanized with carbon dioxide gas and decapitation. The rats were weighed. Trunk blood was collected and placed on the ice until centrifuged and stored at -80°C. Dissected organs, including liver, were weighed, wrapped in foil, frozen in liquid nitrogen and then stored at -80°C. A portion of liver was placed in embedding medium for frozen tissue specimens (OCT) in an embedding mould and frozen in a dry ice-ethanol bath and then stored at -80°C.

Preparation of Liver Protein Samples

Frozen liver tissue (40 mg/per sample) was ground in liquid nitrogen and the powder dissolved in 3 times sample buffer (30 µl per sample of 30% glycerol, 3% SDS, 0.1875 M Tris-HCl pH 6.8). After waiting for 20 minutes, the solution was clarified by centrifugation (13000 rpm) for 20 minutes. The protein supernatant was transferred to a clean tube.

An aliquot of the protein lysate was assayed using the BCA protein assay to determine the protein content of the samples. Ten µl of the blank, protein standards and unknown diluted samples were pippetted in triplicate onto a 96 well plate. The BCA™ Protein Assay reagents (Thermo Scientific) were mixed (50 parts Reagent A and 1 part Reagent B). The total volume of reagents required was calculated using a volume of 200

μL added to each well. Two hundred μL of the mixed reagent was added to the 96 well plate. The plate was sealed with Parafilm to avoid evaporation and incubated at 37°C for 30 minutes. After adjusting the template of microplate reader, the plate was read at 550 nm on a THERMO max micro-plate reader. The protein concentration of the unknowns was determined from the standard curve and adjusted for dilution factor. This information was used to calculate the volumes of the samples to be loaded onto the gels for analysis by Western blotting.

Western Blotting

Western blotting was used determine protein levels of hepatic lipid droplet proteins. In brief, an aliquot of the protein lysate (10 μg protein per lane) was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 10% acrylamide gel composition) and the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting. For immunoblotting, a primary antibody binds to the target protein, and a secondary antibody which binds to the first antibody is used for detection. The antibodies are diluted in a buffer and blocking solution is used to prevent nonspecific binding to the membrane.

Samples were boiled in a microwave for five minutes in preheated water, and then 0.5 μl Bromophenol blue and 0.5 μl β-mercaptoethanol were added to each sample. Equal amounts of protein samples (10 μg protein; 10-15 μL volumes) were then loaded onto the SDS polyacrylamide gel. The protein molecular weight marker, a loading control (eEF2) and an intensity control sample (same sample loaded on each gel) were also loaded. Electrophoresis was conducted at 20 miliampere per gel for around 60-70

minutes. The protein was transferred from the gel to PVDF membrane at 100 volts, regardless of the number of gels. Afterwards, the membrane was stored in 1× Tris-buffered saline with Tween-20 (TBST) and blocked with 3% BSA in TBST for 60 minutes at room temperature with constant agitation. This was followed by incubation with diluted primary antibody (Table 2) for 60 minutes. The membrane was washed three times for 5 minutes with 1× TBST. The membrane was then incubated with diluted secondary antibody for 60 minutes at room temperature with constant agitation. The membrane was washed three times for five minutes with TBST to remove any non-specific antibody. The membrane was then saturated with ECL Western blotting detection luminescent reagent (Amersham GE Healthcare) and exposed to film. The developed film provides a visual record of the location and relative amount of the protein. The mass of the protein is determined by comparison with a protein molecular weight standard. A loading control (eEF2) was used to verify equal protein loading on each lane. The intensity control sample was used to standardize for different intensities on the different gels. The bands were scanned with a GC-800 calibrated densitometer and results were expressed as arbitrary units.

Number and Size of Lipid Droplets in Hepatic Tissue (Histochemistry and Image Analysis)

a) Preparation of H&E Stained Liver Sections

For microscopic observation of lipid droplets in liver cells, sections of 4-5 µm were prepared. The thickness of the samples was consistent on all slides. Two sections

Table 2: Antibodies and Applications

Primary Antibody[1]	Secondary Antibody[2]	Application	Dilution	Size(kDa)	Catalog#	Company	Function
Adipophilin	Mouse	Western Blotting	1:1500 (tissue) 1: 1000 (cell culture)	48	651102	Progen	Adipophilin protein
Perilipin	Goat	Western Blotting	1:1000	57	47319	Santa Cruz Biotechnology	Perilipin protein
eEf2	Rabbit	Western blotting	1:1000	100	2332	Cell Signaling	Loading control

[1]Primary antibodies diluted with 3% BSA-TBST; membranes incubated in primary antibodies for 1 hour.
[2]Secondary antibodies diluted with 1% BSA-TBST; membrane incubated in secondary antibodies for 1 hour

The sections were fixed in 37% (w/w) formaldehyde solution for 10 minutes, and then washed with PBS 3 times for 10 minutes and once with water for one minute. The slides were then immersed in Harris Modifed Hematoxylin for three minutes and rinsed with deionized water. After rinsing, they were placed in tap water for 5 minutes to develop the stain. The slides were then dipped in the acid ethanol 10 times (acid ethanol = 1 mL HCl + 400 mL 70% ethanol) for destaining, rinsed with tap water twice for one minute each time and then rinsed in deionized water for 2 minutes. Slides were placed in eosin (3 grams eosin in 300 ml double distilled water) for 30 seconds once and then immersed in 95% ethanol 3 times for 5 minutes each time. Dehydration was completed by placing the slides in 100% ethanol 3 times for 5 minutes each time. Finally, they were placed into xylene 3 times for 15 minutes each time. Cover slips were put on each slides using Permount (Permanent Mounting Medium). This was done by adding a drop of Permount on each slide, placing the cover slip at an angle and then letting it fall gently on the slide, taking care that no bubbles are trapped.

b) Image Capturing in the Visible Light Spectrum with a Digital Camera

This technique was used for analysis, visual comparison and observation of the lipid droplets. After placing the samples on the stage of the microscope (20× objective; Olympus U-UCD-JAPAN, Markham, ON), photographs were taken with a digital camera (Olympus BH2-RFCA, Carsen Group Inc, Markham, ON). The images were saved on the computer as TIFF files.

For this study, the data were obtained from six rats per treatment group and five slides were imaged per rat. There were five dietary treatment groups in the study, thus 5 × 6 × 5 = 150 sections were analyzed in total.

c) Using Image J Program to Analyze the Slides

Image J v I.34s was used for quantification of the lipid droplets from the microscope images. Image J is an imaging program developed at the National Institute of Health (Rasband,WS, ImageJ, US National Institues of Health, Bethesda, MD, http: www. rsb.info.nih.gov/ij/, 1997-2006). Using this software, lipid droplet areas were selected with the provided tools. The area of the lipid droplets was measured and converted to units of μm^2. Four selected grids were used for data collection on all pictures on the grids, with 4 additional grids selected in case one or more of the original grids was unsuitable. As well, the number of lipid droplets in the four selected grids was recorded.

Cell Culture Study

Induction of Lipid Droplets in H4IIE Hepatoma Cells

A method for *in vitro* model of lipid accumulation has been developed in the laboratory by D. Stringer (personal communication) and remains unpublished. The method uses addition of oleic acid (OA) to the culture media to induce excess lipid accumulation in H4IIE hepatoma, a cell line that is an established model for growth and metabolic processes. These cells contain only a few lipid droplets under the standard culture conditions, however, supplementation of growth medium with 400 μM OA for 24 hours enhances lipid droplet formation in quiescent cells.

The H4IIE cells were cultured in 12-well dishes containing 10% Fetal Bovine Serum (FBS, GIBCO) in Alpha Minimum Essential Medium Eagle (α-MEM, GIBCO) until 80-90% confluency was reached. Quiescence was achieved by placing the cells into serum-free medium for 3 days. To determine the effects of PPAR, LXR and FXR agonists on lipid droplet formation and lipid droplet proteins (adipophilin and perilipin), the cells were pre-treated with the various agonists for 10 minutes before addition of 400 μM OA (Sigma) for 24 hours to induce lipid droplet formation. The agonists and their final concentrations were 100 μM WY14643 (PPAR α agonist), 10 μM Rosiglitazone (PPARγ agonist), 1 μM GW0742 (PPARδ agonist), 1 μM GW 3965 (LXR agonist), and 1 μM GW4064 (FXR agonist). Null treated cells had no agonist or OA treatment.

The OA added to the cultured cells was in a solution and bound to bovine serum albumin (BSA). The fatty acid supplement (OA) was prepared by adding 200 μl 1 M NaOH to 15.7 ml water and warming the solution to 70°C in a water bath. OA (100 μl

0.89 g/ml concentration) was added to the solution, incubated for 30 minutes and then inverted several times to mix. Then 50 μL 1 M NaOH was added to the fatty acid solution and it was inverted again. The solution was incubated for 5 minutes at 70°C. This step was repeated three times until micelles were no longer visible. To prepare the 5% BSA solution, 5 g BSA was added to 80 ml PBS and gently mixed. The volume was adjusted to 100 ml with PBS. The solution was filter-sterilized and stored at 4°C. The fatty acid complex was added to BSA, at an approximate molar ratio of 8:1. In a culture hood, the BSA/oleate mix was added to 160 ml cell culture medium (pre-warmed to 37°C) in a sterile 250 ml beaker. The solution was sterilized with a bottle-top filter. The final concentration of oleate in this medium was 400 μM.

After 24 hours, digital images of the cells were captured and the cells were harvested as described in the next section.

Preparation of Protein Samples from Cells in Culture & Western Blotting

Media was removed from the wells, and after washing 2 times with PBS, 200 μL of 2× times sample buffer was added to each well. After 10 minutes the samples were transferred to a clean tube, each tube was sonicated and the samples were stored at −20°C. After determining the protein content of the samples, Western blotting was used to quantity the levels of adipophilin using the same procedures for Western blotting as described for liver tissue. For cell culture samples, the antibody dilution was 1:1000 for both adipophilin and eEF2 proteins.

The original goal was to analyze the cell culture samples for perilipin, however, several perilipin antibodies did not work for Western blotting of H4IIE cells. Several

attempts to change factors such as dilution of the perilipin antibody and incubation times did not provide clear bands with little background.

Statistical Analysis

ANOVA was used to determine significant main effects, and Duncan's multiple range tests was used for means testing (SAS 9.1; SAS Institute, Cary, NC) to determine differences among specific groups. The level of significance was set at $P<0.05$. All data are reported as means± SEM.

RESULTS

Animal Study

Final Body Weight

During the 8 week study, the fa CTL rats gained 179 g compared to the fa Base group (Figure 1). The *fa/fa* Zucker rats had a higher final body weight compared to lean Zucker rats. Body weights were 609 ± 19 g for fa Base, 785 ± 12 g for fa CTL, 791 ± 15 g for fa 9-11, 791 ± 20 g for fa 10-12 and 468 ± 11 g for ln CTL. There was no effect of dietary CLA treatments on body weight.

Weekly Body Weight

The *fa/fa* Zucker rats had significantly higher weekly body weights compared to lean Zucker rats (Figure 2). No significant differences were observed with the CLA diets.

Total Feed Intake

The *fa/fa* Zucker rats had a higher total feed intake compared to lean Zucker rats and the amounts for the total feed intake were as follows: 1470 ± 24 g for fa CTL, 1489 ± 37 g for fa 9-11, 1506 ± 37 g for fa 10-12 and 1060 ± 19 g for ln CTL (Figure 3). Dietary CLA isomers did not alter feed intake in *fa/fa* Zucker rats.

Figure 1 - The effect of CLA 10-12 and CLA 9-11 diets on final body weight (g) in *fa/fa* Zucker rats. Data are expressed as mean ± standard error (n=10 per group). Statistical significance (p<0.05) was determined by ANOVA and Duncan's multiple range test. Bars with different letters are significantly different. Abbreviations: Fa Base= *fa/fa* rats at baseline (16 weeks old); fa CTL= rats fed control diet for 8 weeks; fa 9-11= *fa/fa* rats fed 0.4% (w/w) c9, t11-CLA diet for 8 weeks; fa 10-12 = *fa/fa* rats fed 0.4% (w/w) t10, c12-CLA diet for 8 weeks; ln CTL= lean rats fed control diet for 8 weeks.

Figure 2 – The effect of CLA 10-12 and CLA 9-11 diets on weekly body weight (g) in *fa/fa* **Zucker rats.** Data are expressed as mean ± standard error (n=10 per group). Statistical significance (p<0.05) was determined by ANOVA and Duncan's multiple range test. At each time point, symbols with different letters indicate are significant differences. See Figure 1 legend for abbreviations.

Figure 3 - The effect of CLA 10-12 and CLA 9-11 diets on total feed intake (g) in *fa/fa*
Zucker rats. Data are expressed as mean ± standard error (n=10 per group). Statistical
significance (p<0.05) was determined by ANOVA and Duncan's multiple range test. Bars with
different letters are significantly different. See Figure 1 legend for abbreviations.

Weekly Feed Intake

The *fa/fa* Zucker rats had significantly higher weekly feed intake compared to lean Zucker rats (Figure 4). Weekly feed intake was not affected by dietary CLA.

Liver Weight

The *fa/fa* Zucker rats had a higher liver weight compared to lean Zucker rats (Figure 5a). The liver weights were 31.28 ± 1.72 g for fa Base, 34.96 ± 1.23 g for fa CTL, 34.43 ± 1.49 g for fa 9-11, 33.83 ± 1.54 g for fa 10-12 and 13.93 ± 0.56 g for ln CTL. No effects of dietary CLA treatments were observed.

The *fa/fa* Zucker rats had a higher liver weight/body weight compared to lean Zucker rats and a lower liver weight/body weight compared to fa Base (Figure 5b). The liver weight/body weights were 5.15 ± 0.26 g for fa Base, 4.47 ± 0.17 g for fa CTL, 4.30 ± 0.13 g for fa 9-11, 4.29 ± 0.22 g for fa 10-12 and 2.98 ± 0.12 g for ln CTL. There was no effect of dietary CLA treatments.
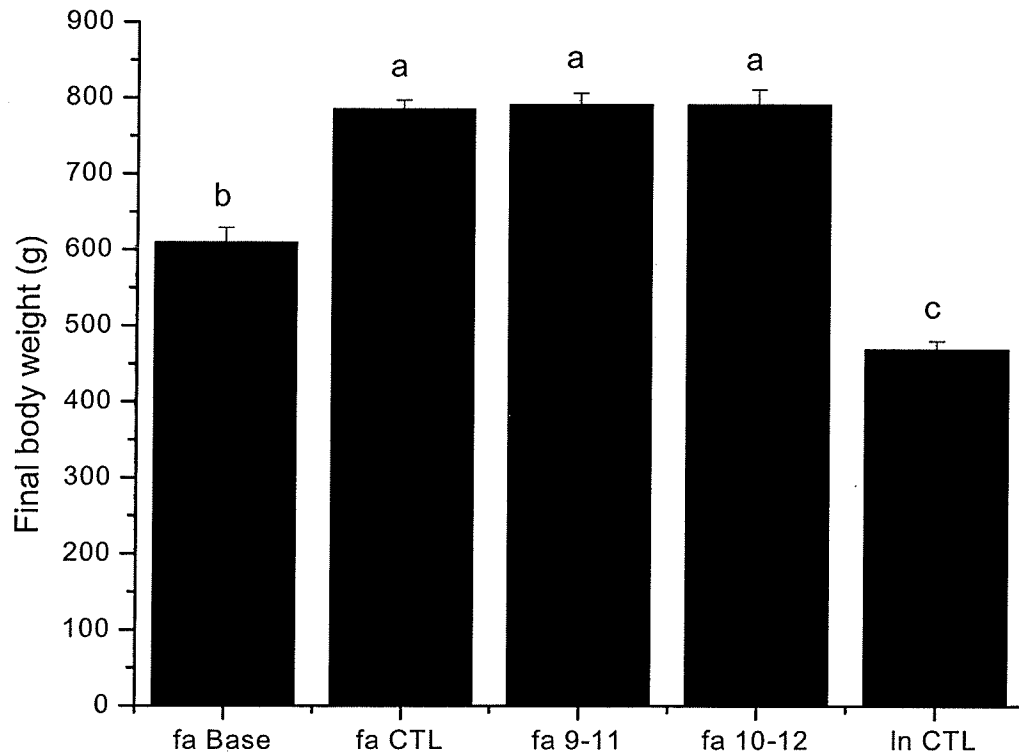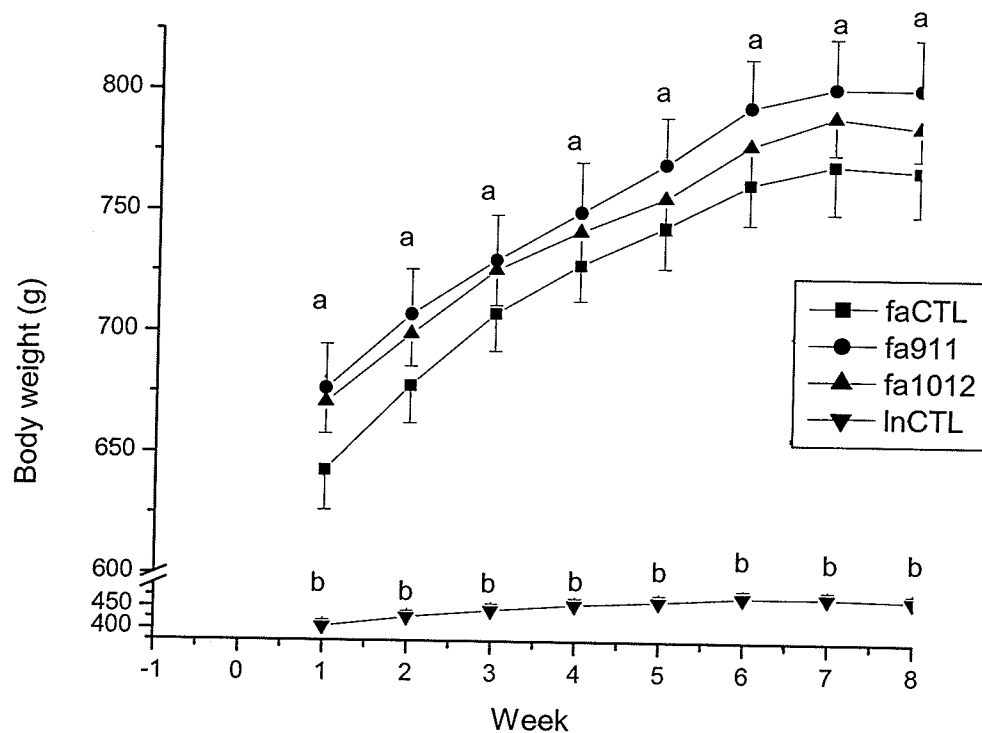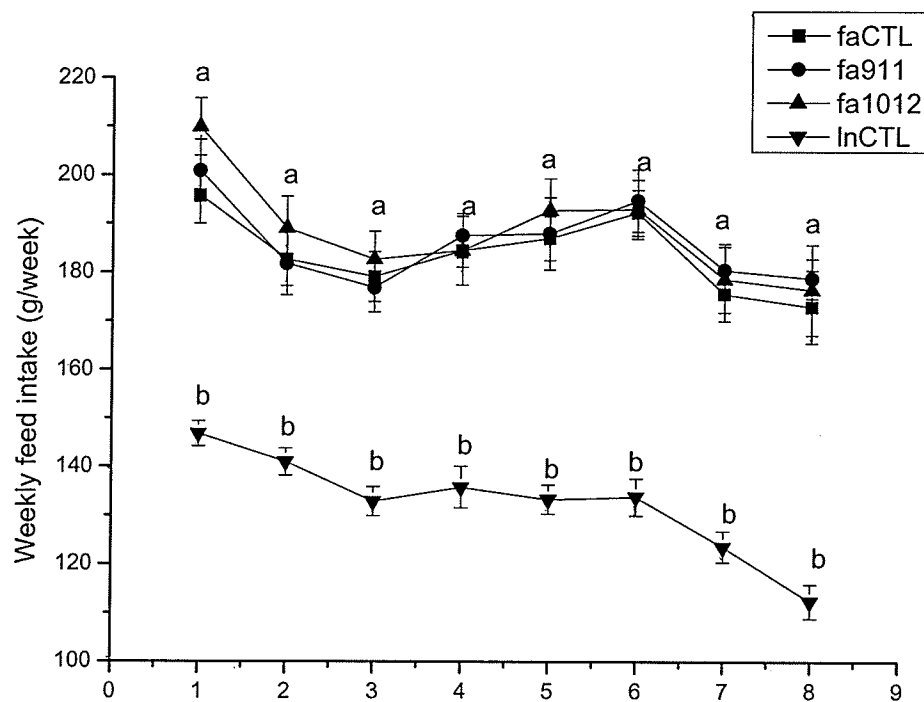
Figure 4 - The effect of CLA 10-12 and CLA 9-11 diets on weekly feed intake (g) in *fa/fa*
Zucker rats. Data are expressed as mean ± standard error (n=10 per group). Statistical
significance (p<0.05) was determined by ANOVA and Duncan's multiple range test. At each
time point, symbols with different letters are significantly different. See Figure 1 legend for
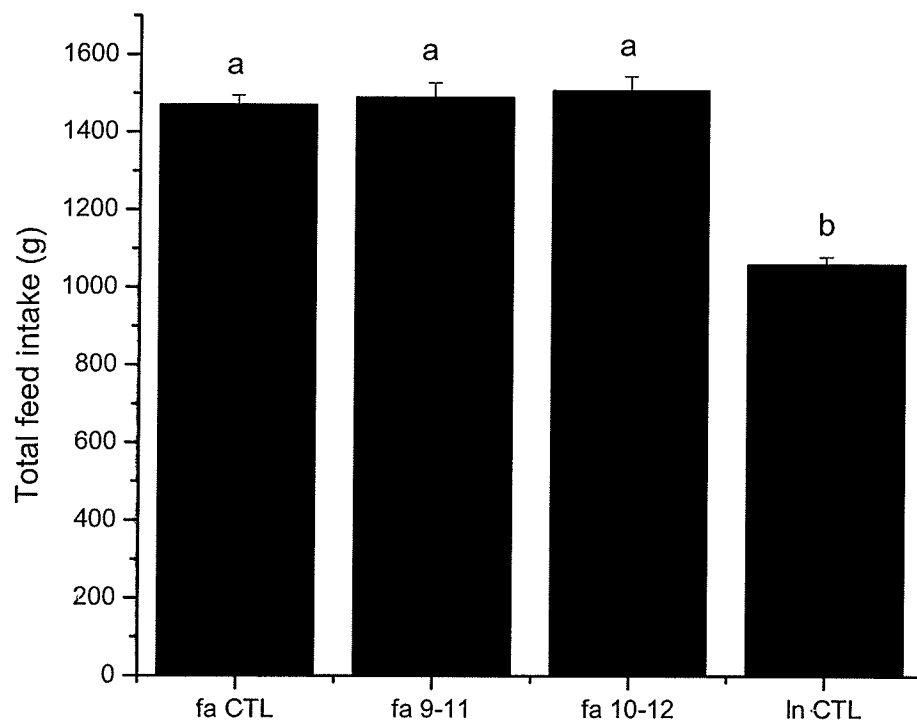abbreviation.

a)
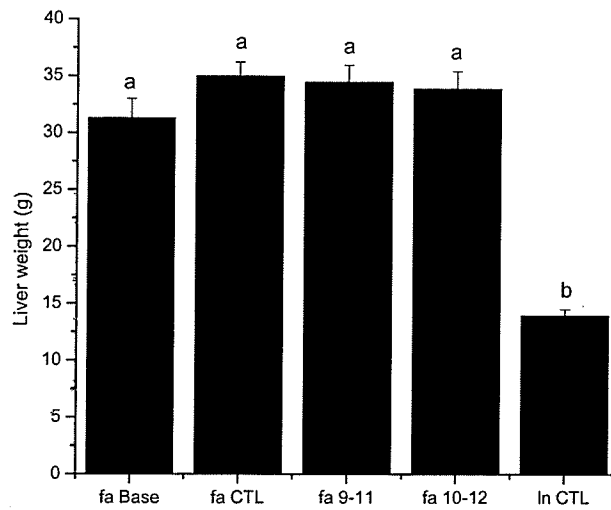
b)

Figure 5 - The effect of CLA 10-12 and CLA 9-11 diets on a) liver weight (g) and b) liver/body weight ratio in *fa/fa* **Zucker rats.** Data are expressed as mean ± standard error (n=10 per group). Statistical significance (p<0.05) was determined by ANOVA and Duncan's multiple range test. Bars with different letters are significantly different. See Figure 1 legend for abbreviations.

Epididymal Fat

At the end of the study period the animals exhibited differences in the amount of epididymal fat (Figure 6a). The fa Base and fa CTL had less epididymal fat compared to fa 10-12. All four *fa/fa* groups had a higher amount of epididymal fat than ln CTL. There were no significant differences within the CLA diet groups. The amount of the epididymal fat was as follows: 18.65 ± 1.28 g for fa Base, 21.35 ± 0.94 g for fa CTL, 22.87 ± 0.89 g for fa 9-11, 21.35 ± 0.94 g for fa 10-12 and 9.69 ± 0.59 g for ln CTL.

The ln CTL had the lowest epididymal fat/ body fat (g/100 g) (Figure 6b). There was no significant difference in the amount of epididymal fat/body weight among fa Base, fa CTL and fa 9-11 groups. The fa 10-12 group had a higher amount of epididymal fat/body weight (g/100 g) compared to fa CTL and ln CTL.

a)
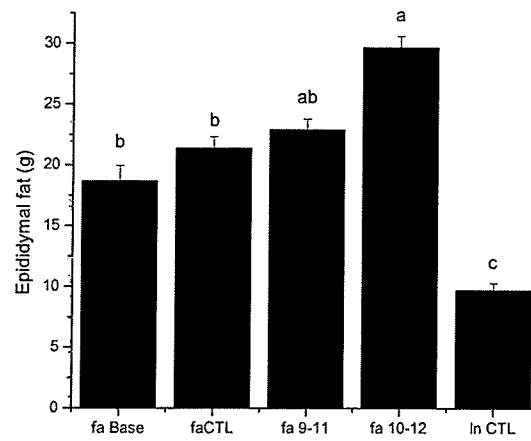
b)
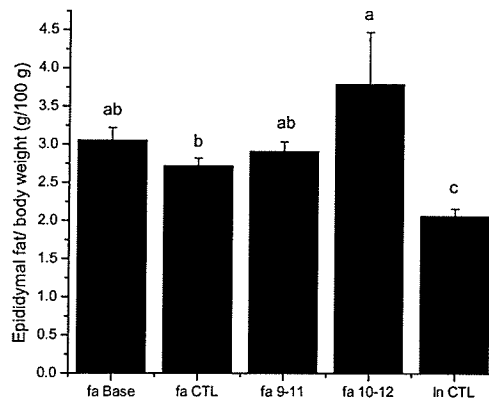
Figure 6 - The effect of CLA 10-12 and CLA 9-11 diets on epididymal fat weight and b) epididymal fat/body weight (g/100 g) in *fa/fa* Zucker rats. Data are expressed as mean ± standard error (n=10 per group). Statistical significance (p<0.05) was determined by ANOVA and Duncan's multiple range test and bars with different letters are significantly different. See Figure 1 legend for abbreviations.

Peri-renal Fat

Peri-renal fat levels were significantly different among diet groups (Figure 7a). The lowest amounts were observed in the ln CTL and the highest amount in the fa 10-12. There were no significant differences among faCTL, fa 9-11 and fa 10-12, but these three groups were significantly different from fa Base and ln CTL. The peri-renal fat amounts were as follows: 37.52 ± 1.73 g for fa Base, 59.42 ± 3.17 g for fa CTL, 63.52 ± 2.46 g for fa 9-11, 72.86 ± 3.27 g for fa 10-12 and 10.26 ± 0.71 g for ln CTL.

Figure 7b shows significant differences between CLA dietary groups regarding the ratio of peri-renal fat/body weight (g/100 g). There were no significant differences between fa CTL and fa 9-11. The fa 10-12 was significantly higher than fa CTL and fa 9-11, and these three groups were significantly higher than fa Base and lnCTL. The ln CTL had less peri-renal fat compared to fa Base. The peri-renal fat/body weight (g/100 g) was as follows: 6.14 ± 0.16 g for fa Base, 7.56 ± 0.38 g for fa CTL, 8.02 ± 0.26 g for fa 9-11, 9.2 ± 0.31 g for fa 10-12 and 2.18 ± 0.12 g for ln CTL.
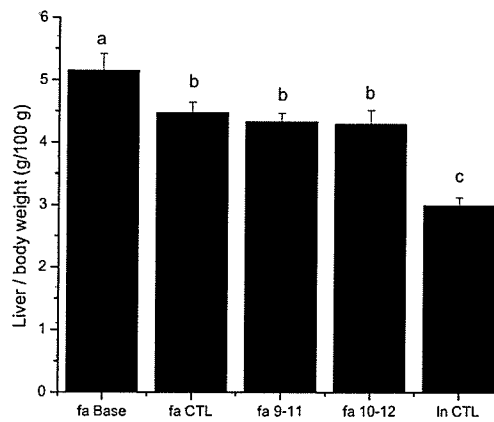
a)
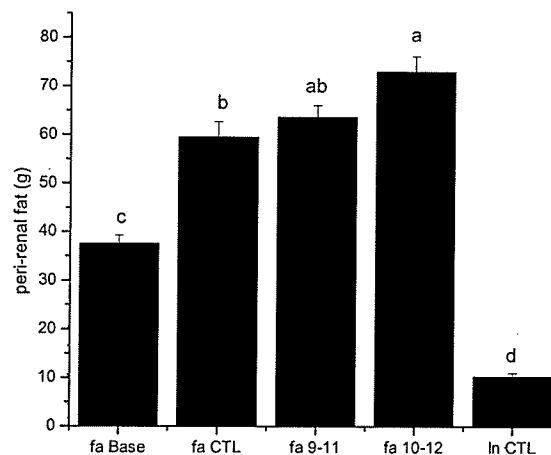
b)

Figure 7 - The effect of CLA 10-12 and CLA 9-11 diets on a) peri-renal fat weight (g) and b) peri-renal fat/ body weight (g/100 g) in *fa/fa* Zucker rats. Data are expressed as mean ± standard error (n=10 per group). Statistical significance (p<0.05) was determined by ANOVA and Duncan's multiple range test. Bars with different letters are significantly different. See Figure 1 legend for abbreviations.

Visceral Fat

The sum of epididymal fat and peri-renal fat pad weighs was used as a measure of visceral fat. The *fa/fa* Zucker rats fed control diet had significantly higher amounts of visceral fat compared to fa Base and ln CTL rats (Figure 8a). The fa 10-12 had significantly more visceral fat than fa CTL and fa 9-11. The amount of visceral fat was higher in fa Base than ln CTL. The amount of visceral fat was as follows: 56.17 ± 2.58 g for fa Base, 80.77 ± 3.87 g for fa CTL, 86.40 ± 2.98 g for fa 9-11, 102.50 ± 6.19 g for fa 10-12 and 19.95 ± 1.27 g for ln CTL.

The amount of visceral fat relative to body weight (g/100 g) was also significantly different among the groups (Figure 8b). The fa 10-12 was significantly higher than other groups. There were no significant differences between fa CTL and fa 9-11 or between fa CTL and fa Base. The lowest amount of visceral fat was seen in ln CTL. The corresponding amounts were as follows: 9.2 ± 0.23 g for fa Base, 10.27 ± 0.45 g for fa CTL, 10.97 ± 0.34 g for fa 9-11, 12.99 ± 0.81 g for fa 10-12 and 4.24 ± 0.21 g for ln CTL.

a)

b)

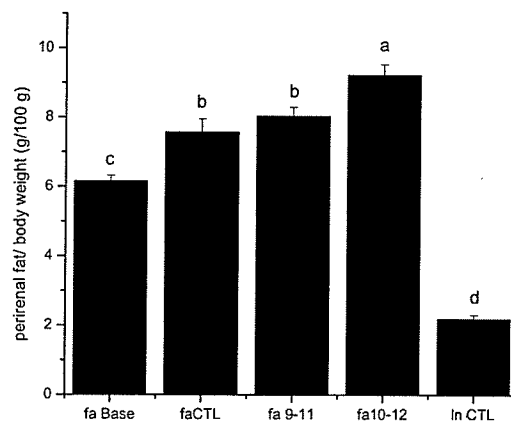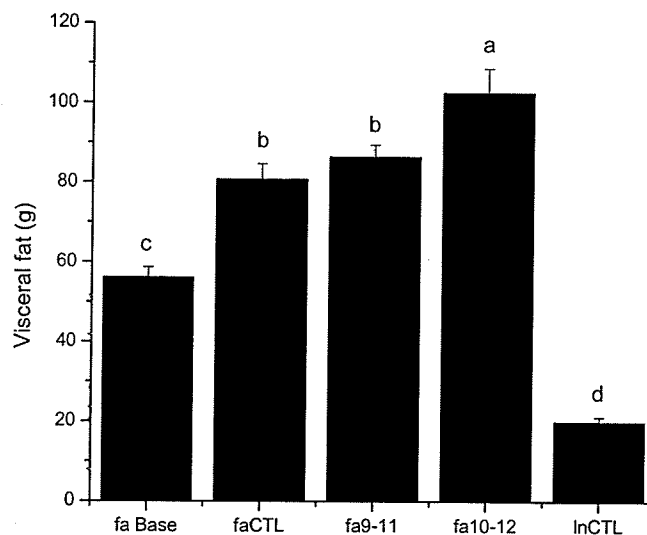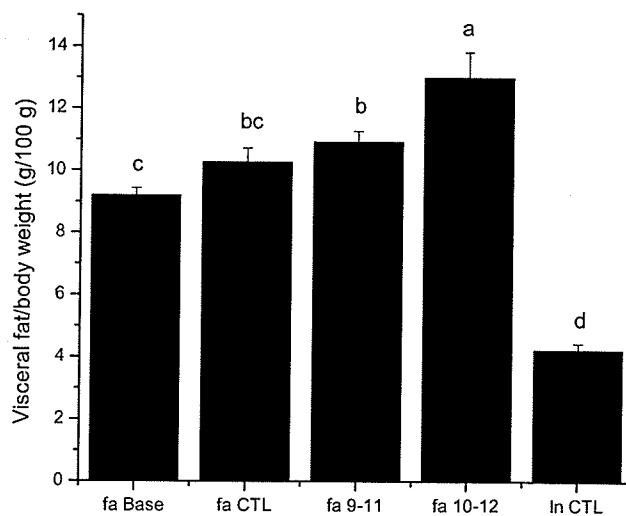**Figure 8 - The effect of CLA 10-12 and CLA 9-11 diets on a) visceral fat (g) and b) visceral fat /body weight (g/100 g) in** *fa/fa* **Zucker rats.** Data are expressed as mean ± standard error (n=10 per group). Statistical significance (p<0.05) was determined by ANOVA and Duncan's multiple range test. Bars with different letters are significantly different. See Figure 1 legend for abbreviations.

Lipid Accumulation in Liver Sections

Liver sections were stained with H&E to visualize the amount of lipid accumulation. By visual inspection, the fa CTL had an abundance of large lipid droplets indicating considerable lipid accumulation (Figure 9). The fa 9-11 and fa 10-12 appeared to have less lipid than the fa CTL. The fa Base had less lipid accumulation than the fa 9-11 and fa 10-12. There was negligible lipid accumulation in the ln CTL.

Hepatic Lipid Droplet Numbers

The number of lipid droplets in the liver tissue was quantified. As shown in Figure 10 there were no significant differences in lipid droplet numbers between the CLA dietary groups. The ln CTL had significantly fewer lipid droplets than all other dietary groups. The lipid droplet numbers in liver cells were as follows: 331 ± 68.37 for fa Base, 453 ± 37.81 for fa CTL, 399 ± 38.07 for fa 9-11, 382 ± 57.31 for fa 10-12 and 45 ± 22.93 for ln CTL.

Hepatic Lipid Droplet Area

The area of lipid droplets in the liver tissue was also quantified. As shown in Figure 11 there were significant differences in lipid droplet area between the CLA dietary groups. The fa CTL had a significantly larger lipid droplets than other groups. The ln CTL had a significantly lower hepatic lipid droplet area than other dietary groups. The lipid droplet areas in liver cells were as follows: $5.451 \times 10^{-5} \pm 1.4 \times 10^{-5}$ for fa Base, $3.323 \times 10^{-4} \pm 2.2 \times 10^{-4}$ for fa CTL, $6.777 \times 10^{-5} \pm 1.1 \times 10^{-5}$ for fa 9-11, $4.311 \times 10^{-5} \pm 7.3 \times 10^{-6}$ for fa 10-12 and $1.822 \times 10^{-5} \pm 5.4 \times 10^{-6}$ for ln CTL. The largest lipid droplets were observed in the fa CTL group.
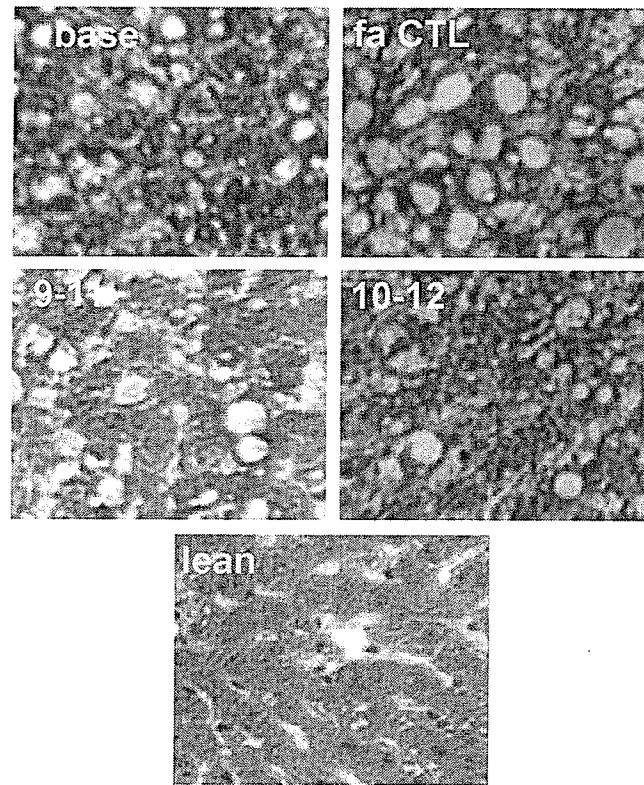
Figure 9 – Images of liver sections from *fa/fa* Zucker rats and lean rats showing lipid accumulation. Liver sections were stained with H&E. The unstained (white) areas represent lipid in the cells. By visual inspection of the images, lipid accumulation was the lowest in the ln CTL and greatest in fa CTL. See Figure 1 legend for abbreviations.
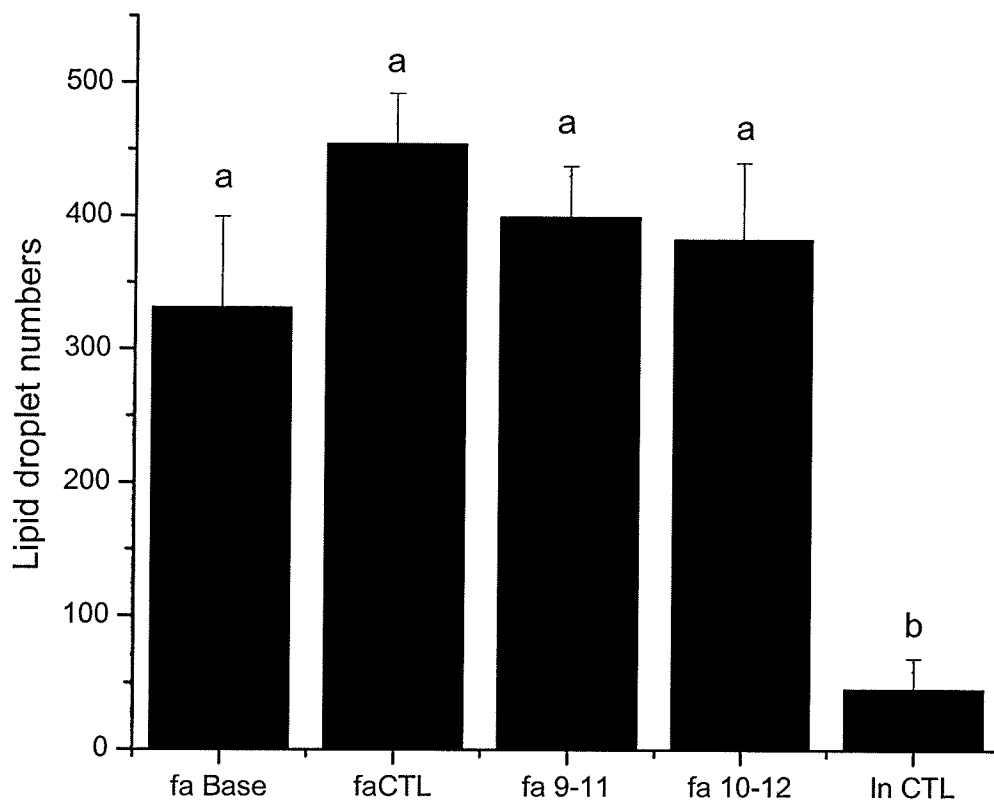
Figure 10 - The effect of CLA 10-12 and CLA 9-11 diets on hepatic lipid droplet numbers.
Data are expressed as mean ± standard error (n=6 rats per group). Statistical significance (p<0.05)
was determined by ANOVA and Duncan's multiple range test. Bars with different letters are
significantly different. The higher amounts of hepatic lipid in the fa CTL could be located as
extracellular or intracellular droplets. The tissue was therefore examined microscopically, and
intracellular lipid droplets were present in the hepatocytes of the *fa/fa* rats. Very few droplets
were seen in the ln CTL. See Figure 1 legend for abbreviations.
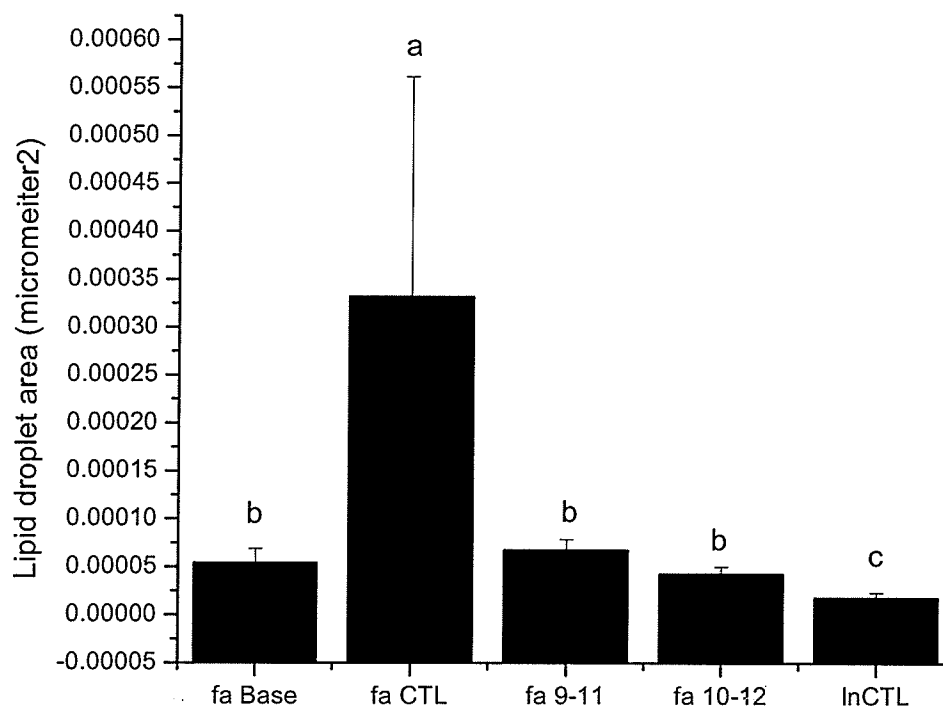
Figure 11 - The effect of CLA 10-12 and CLA 9-11 diets on hepatic lipid droplet area. Data are expressed as mean ± standard error (n=6 rats per group). Statistical significance (p<0.05) was determined by ANOVA and Duncan's multiple range test. Bars with different letters are significantly different. The higher amounts of hepatic lipid in the fa CTL could be located as extracellular or intracellular droplets. The tissue was therefore examined microscopically, and intracellular lipid droplets were present in the hepatocytes of *fa/fa* rats. Very few droplets were seen in the ln CTL. See Figure 1 legend for abbreviations.

Adipophilin Lipid Droplet Protein in Liver Tissue

To determine the effect of CLA on the lipid droplet protein adipophilin in liver tissue of *fa/fa* Zucker rats, Western blotting was employed (Figure 12). One of the samples was repeated on all gels to control for loading variability. eEF2 was employed as the internal loading control. The ratio of adipophilin/eEF2 was used to account for loading variation. Neither of the CLA isomers affected the level of eEF2 protein. The fa Base, fa CTL and fa 9-11 exhibited significantly higher amounts of adipophilin compared to fa 10-12 and ln CTL. Treatment with 10-12 CLA significantly decreased adipophilin protein levels in *fa/fa* rats to the same level as ln CTL. The adipophilin protein levels in liver tissue were as follows: 3.35 ± 0.71 for fa Base, 4.64 ± 0.88 for fa CTL, 3.97 ± 0.69 for fa 9-11, 0.53 ± 0.87 for fa 10-12 and 0.30 ± 0.92 ln CTL. The presence of high lipid droplet levels in the liver of fa CTL in comparison with ln CTL suggests that these proteins are responsible for organizing these structures they are responsive to c10,t12 CLA.
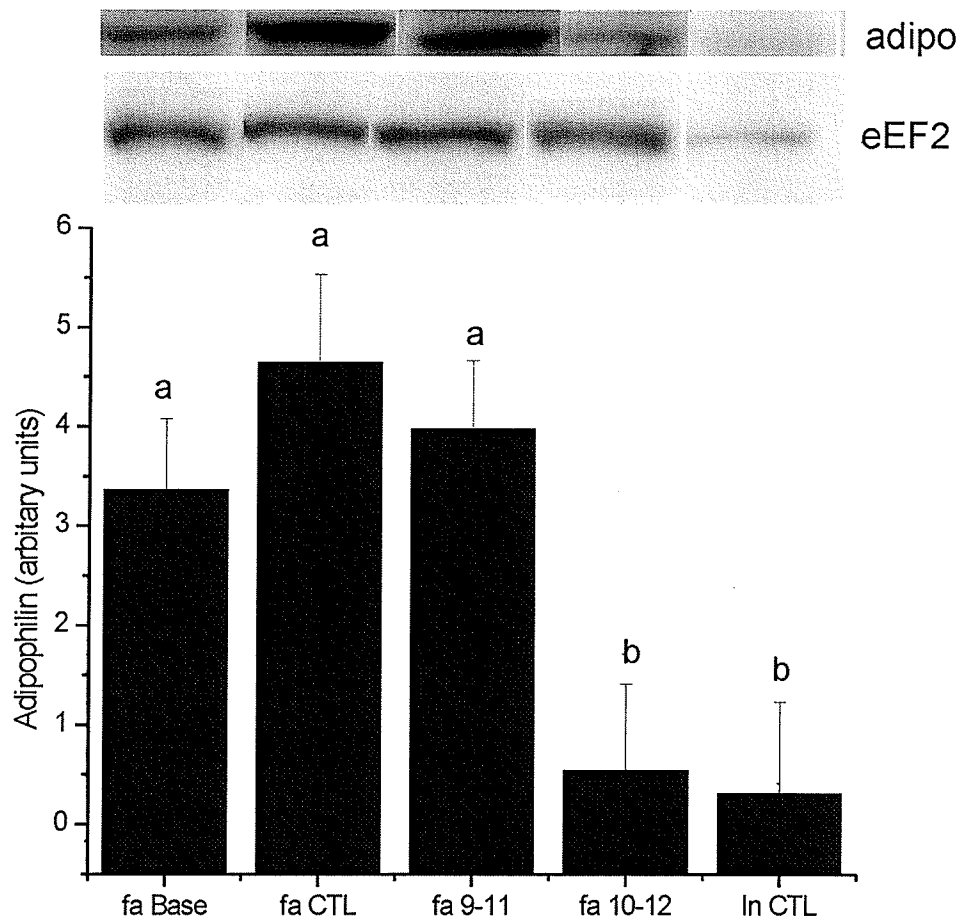
Figure 12 - The effect of CLA 10-12 and CLA 9-11 diets on adipophilin lipid droplet protein in liver tissue of *fa/fa* Zucker rats. Adipophilin protein levels were assessed by Western blot analysis. The upper panels are representative blots for adipophilin and eEF2, and the lower panel is the corresponding graph obtained by scanning densitometry of the bands in arbitrary units. Data are expressed as mean ± standard error (n=5 for fa CTL and fa 10-12, n=6 for ln CTL, n=8 for fa Base and fa 9-11). Statistical significance (p<0.05) was determined by ANOVA and Duncan's multiple range test. Bars with different letters are significantly different. See Figure 1 for legend abbreviations.

Perilipin Lipid Droplet Protein in Liver Tissue

To determine the effect of CLA isomers on the lipid droplet protein perilipin in liver tissue of *fa/fa* Zucker rats, Western blotting was employed (Figure 13). One of the samples was repeated in all gels to control for loading variability. eEF2 was employed as the internal loading control. The ratio of perilipin/eEF2 was used to account for loading variation. Neither of the CLA isomers affected the level of eEF2 protein. The fa CTL, fa 9-11 and fa 10-12 exhibited significantly lower amounts of perilipin compared to ln CTL. Perilipin protein levels in fa CTL, fa 9-11 and fa 10-12 are lower than fa Base, but the differences were not significant. There was no significance difference between fa Base and ln CTL. The perilipin protein amount in liver cells was as follows: 0.86 ± 0.20 for fa Base, 0.57 ± 0.20 for fa CTL, 0.51 ± 0.19 for fa 9-11, 0.45 ± 0.20 for fa 10-12 and 1.05 ± 0.20 for ln CTL.
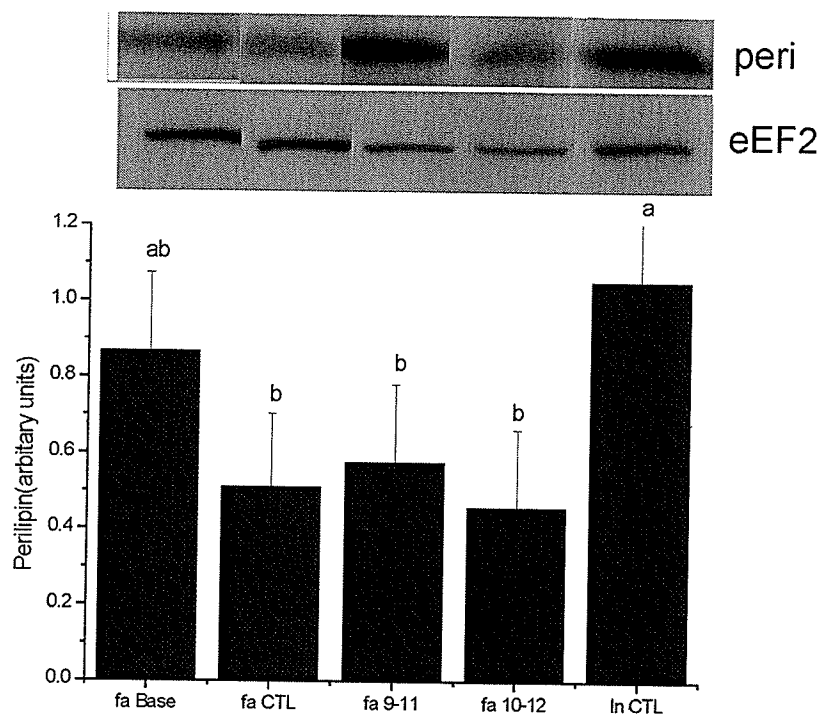
Figure 13 - The effect of CLA 10-12 and CLA 9-11 diets on perilipin lipid droplet protein in liver tissue of *fa/fa* Zucker rats. Perilipin protein levels were assessed by Western blot analysis. The upper panels are representative blots for perilipin and eEF2, and the lower panel is the corresponding graph obtained by scanning densitometry of the bands in arbitrary units. Data are expressed as mean ±standard error (n=7 for fa Base and fa 10-12 and fa 9-11, n=8 for ln CTL, n=9 for fa CTL). Statistical significance (p<0.05) was determined by ANOVA and Duncan's multiple range test. Bars with different letters are significantly different. See Figure 1 for legend abbreviations.

Cell Culture Study

Effects of PPAR, LXR and FXR Agonists on Lipid Accumulation

Figure 14 shows images of for lipid accumulation and lipid droplets in H4IIE cells treated with PPAR, LXR and FXR agonists. Cells were pre-treated with each agent individually for 10 minutes followed by oleic acid (OA) and the agonist treatment for 24 hours. The agonists and their final concentrations were 100 μM WY14643 (PPAR α agonist), 10 μM Rosiglitazone (PPARγ agonist), 1 μM GW0742 (PPARδ agonist), 1 μM GW 3965 (LXR agonist), and 1 μM GW4064 (FXR agonist). Null treated cells received no agonist or OA treatment.

As shown in the images, the number of lipid droplets varied in each group. There were some lipid droplets with OA treatment but this lipid accumulation was prevented by the PPARα and PPARγ agonists (WY14643 and rosiglitazone treatments, respectively). Cells treated with the LXR and FXR agonists (GW3965 and GW4064, respectively) had the most lipid droplets. Lipid droplets in the PPARδ (GW0742) treatment were similar to OA treatment alone. The null group had the lowest amount on lipid droplets compared to other groups.
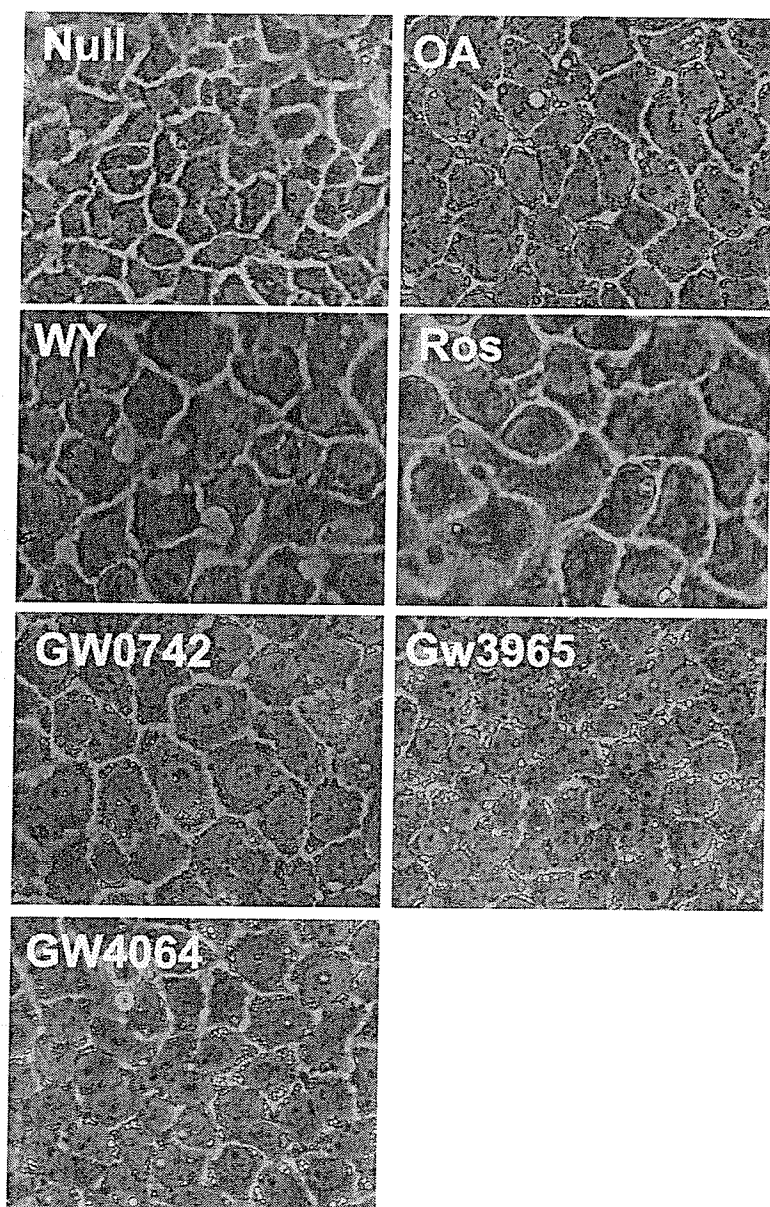
Figure 14 - Images of H4IIE cells treated with PPAR, LXR and FXR agonists. H4IIE cells were untreated (null) or treated with oleic acid (OA), PPARα agonist WY14643 + OA (WY), PPARγ agonist Rosiglitazone + OA (Ros), PPARδ agonist GW0742 + OA (GW0742), LXR agonist GW 3965 + OA (GW3965) or FXR agonist GW4064 + OA (GW4064) as described in the Methods. As shown in the images, there were some lipid droplets with OA treatment but this lipid accumulation was prevented by the PPARα and PPARγ agonists (WY14643 and rosiglitazone treatments, respectively). Cells treated with the LXR and FXR agonists (GW3965 and GW4064, respectively) had the most lipid droplets. Lipid droplets in the PPARδ (GW0742) treatment were similar to OA.

Effects of PPAR, LXR and FXR Agonists on Adipophilin

To determine the effect of PPAR, LXR and FXR ligands on adipophilin protein in H4IIE cell culture model, Western blotting was employed (Figure 15). The cells were pretreated with the agonists for 10 minutes before treatment with oleic acid (OA) and agonists for 24 hours. The treatments were null (non treated control), OA , OA + LXR agonist GW 3965, OA+ FXR agonist GW4064, OA+ PPARδ agonist GW0742, OA +PPARγ agonist Rosiglitazone, and OA +PPARα agonist WY14643. One of the samples was repeated in all gels to control for loading variability. eEF2 was employed as the internal loading control. The ratio of adipophilin/eEF2 was used to account for loading variation. None of the ligands affected the level of eEF2 protein.

The cells treated with the PPARγ agonist rosiglitazone and the cells treated with the PPARα agonist WY14643 exhibited significantly lower amounts of adipophilin protein compared to cells treated with the LXR agonist GW 3965 or FXR agonist GW4064. In fact, the adipophilin levels in the rosiglitazone and WY14643 cells were no different from the null cells. Although the adipophilin protein levels in cells treated with rosiglitazone or WY14643 were lower than the PPARδ agonist GW0742 or the OA treatment alone, the differences were not significant.

The adipophilin protein levels in H4IIE cells were as follows: 2.83 ± 1.08 for null, 6.61 ± 1.07 for oleic acid, 8.29 ± 1.49 for GW 3965, 7.51 ± 1.49 for GW4064, 6.20 ± 1.49 for GW0742, 3.54 ± 1.49 for rosiglitazone and 3.91 ± 1.51 for WY14643.
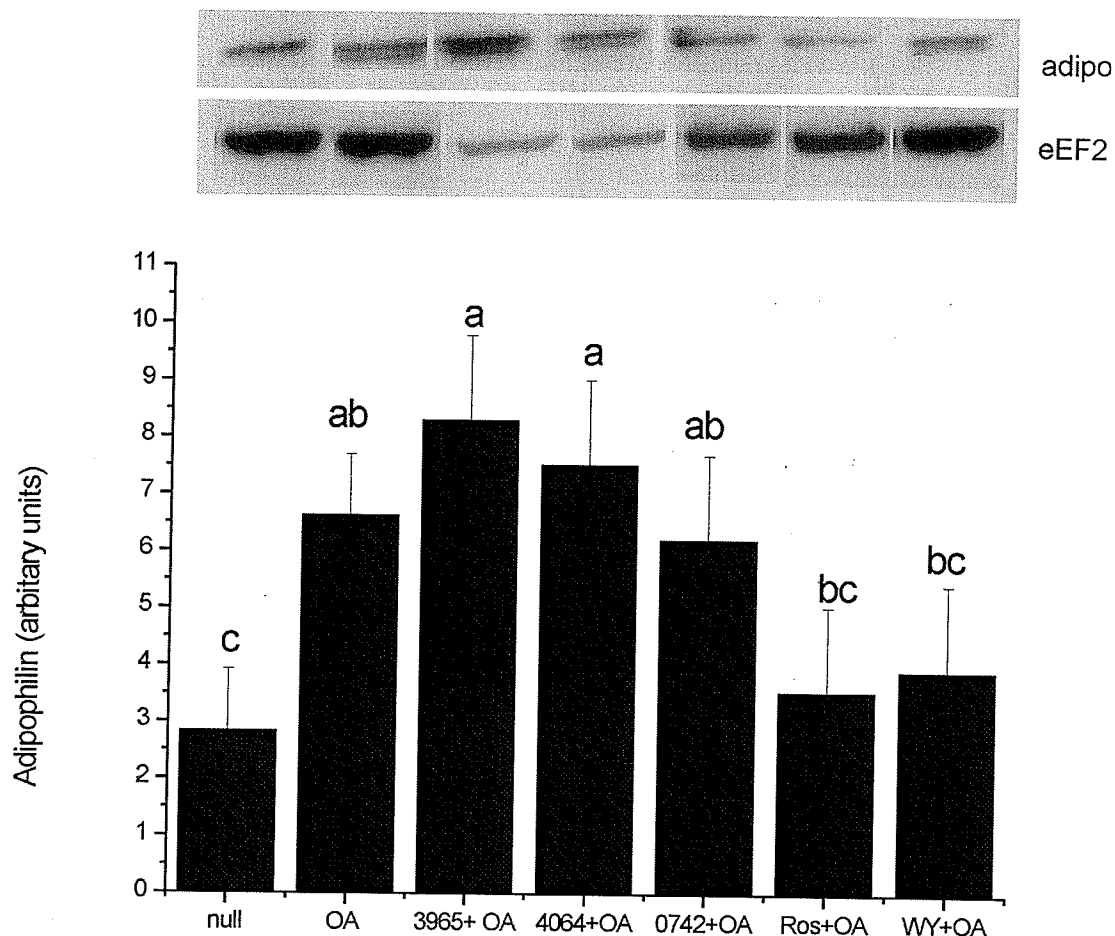
Figure 15 - The effect of PPARs agonist treatments on adipophilin protein in H4IIE cells.
H411E cells were pre-treated with each agonist individually for 10 minutes and then oleic acid
(OA) for 24 hours. Adipophilin protein levels were assessed by Western blot analysis. The upper
panels are representative blots for adipophilin and eEF2, and the lower panel is the corresponding
graph obtained by scanning densitometry of the bands in arbitrary units. Data are expressed as
mean ±standard error for n=3; a duplicate experiment showed similar results. Statistical
significance (p<0.05) was determined by ANOVA and Duncan's multiple range test and bars
with different letters are significantly different. Null is non-treated controls (no agonist and no
OA), OA (oleic acid), 3965+OA [Oleic acid + LXR agonist (1 uM GW 3965)], 4064+OA [oleic
acid + FXR agonist (1μM GW4064)], 0742+OA [oleic acid + PPARδ agonist (1μM GW0742)],
Ros+OA [oleic acid + PPARγ agonist (10 μM Rosiglitazone)], WY+OA [oleic acid +PPARα
agonist (100 μM WY14643)].

DISCUSSION

The current study investigated the effects of CLA isomers on the lipid droplet proteins adipophilin and perilipin, and the size and numbers of lipid droplets in the liver of *fa/fa* Zucker rats. In addition, a cell culture study was used to determine the effects of PPAR, LXR and FXR agonists on adipophilin in H4IIE cells. We found that dietary t10,c12 CLA increased peri-renal and epididymal adipose mass in *fa/fa* Zuckers rats without changes in feed intake or body weight, and at the same time, reduced hepatic steatosis without affecting liver weight. Thus, the reduction in hepatic steatosis did not improve overall adiposity. Our study supports that t10,c12 CLA is the active isomer responsible for changes in adipose mass and liver lipid content. The major findings were that t10,c12 CLA treatment reduces hepatic steatosis in *fa/fa* rats, and this was associated with a reduction in adipophilin but not perilipin in liver tissue. Both the fa 9-10 and fa 10-12 groups had a reduction in the size of lipid droplets, but there was no change in number of lipid droplets compared to fa CTL. The cell culture study indicated that reductions in lipid accumulation and adipophilin levels in H4IIE cells are mediated by PPARγ and PPARα, but not PPARβ, LXR or FXR. These results will be discussed in further detail in the following sections.

Animal Study

Feed Intake and Body Weight

In the current study, the total feed intake was higher in *fa/fa* Zucker rats compared to ln CTL rats (Figure 3). There were no significant differences between CLA dietary

groups and fa CTL for total feed intake. As expected, final body weight was higher in *fa/fa* Zucker rats compared to ln CTL and fa Base counterparts (Figure 1). However, there was no significant difference between fa CLA dietary groups and fa CTL group for final body weight. Our data also indicated that there was no effect of CLA supplementation on weekly body weight (Figure 2). In all cases, however, the weekly body weights were higher in *fa/fa* Zucker rats compared to lean control rats.

The findings for feed intake and body weight of this study agree with other research using *fa/fa* Zucker rats as the study model. The current study used 17 week old *fa/fa* Zucker rats provided with the dietary treatments for 8 weeks (i.e. the rats were 25 weeks old at the end of the study). In previous work from our laboratory, Noto et al. (2007a&b) also found no differences in total body weight and total feed intake when 6 week old *fa/fa* Zucker rats were fed a diets containing a 1.5% CLA mixture for 8 weeks. Stringer (2006) investigated the effects of 0.4% c9,t11 CLA or 0.4% t10,c12 CLA isomers in 6 week old *fa/fa* Zucker rats for a duration of 8 weeks. The results showed that total feed intake was lowest in the lnCTL fed group, and that *fa/fa* rats fed t10,c12 CLA had lower feed intake compared to the *fa/fa* rats fed the control or c9,t11 diet. Final body weight was lowest in the lean groups, however, there was no different among the *fa/fa* treatment groups. In another study conducted by Nagao et al. (2005), there were no significant differences in feed intake, final body weight or body weight gain when 6 week old male *fa/fa* Zucker rats were fed a 1% CLA mixture for 8 weeks. These studies using *fa/fa* Zucker rats as the model show that CLA mixtures or CLA isomers do not alter body weight and only one of the three studies showed that the t10,c12 CLA group had a lower feed intake.

Effects of CLA have also been studied in other rat models for obesity, insulin resistance and diabetes. Ryder et al. (2001) fed 7 week old ZDF rats with 1.5% CLA either as a CLA-rich butter (primarily c9,t11) or as a 50:50 synthetic CLA mixture (containing both c9,t11 and t10,c12 CLA) for 14 days. The results showed food intake was similar among rats fed the control and c9,t11 diets until day 10, and then food intake was significantly increased in the c9,t11 group and remain higher to the end of study. The food intake of the 50:50 group was similar during the first four days of the study, and then it significantly decreased on day five and remained lower until the end of the study. Final body weight was significantly higher in the ZDF rats in the control and c9,t11 CLA groups compared to the 50:50 mixture group. This study may have found effects of CLA isomers on food intake and body weight because they used diabetic ZDF rats, a higher dose of CLA and a short term study of only 14 days, compared to the present study which used obese insulin-resistant *fa/fa* Zucker rats fed 0.4% CLA isomers for 8 weeks. However, in the present study there was no difference in weekly feed intake or body weights at weeks 1 and 2.

Purushotham et al. (2006) fed 4 week old male Wistar rats were fed a high fat diet (20% fat by weight) for 4 weeks to induce obesity and hepatic steatosis, and this was followed by a low fat diet (6.5% fat by weight) containing control oil (soybean oil) or a 1.5% CLA mixture for an additional four weeks. Their results showed that the CLA diet during the low fat experimental period did not change body weights or food intake compared to the control diet without CLA. This study shows that CLA does not affect feed intake or body weight in a diet-induced model of obesity, similar to the *fa/fa* Zucker rats used in the present study which have a single gene mutation in the leptin receptor.

Adipose Tissue

In this study, visceral fat was defined as peri-renal plus epididymal adipose tissue. Peri- renal fat is the fat located in peritoneal cavity that surrounds the kidneys. It has a protective role. Epididymal fat is attached to the epididymis in male rats. Increases in visceral fat cause central adiposity in obese animals.

Comparison of the results in this study is based on the adipose weight relative to body weight (g/100 g body weight) to take into account differences in body weight. The results of this study showed that there were no significant differences in visceral or epididymal fat in fa Base versus fa CTL at the end of the study period (Figures 6 and 8), but peri-renal fat was lower in fa Base compared to fa CTL (Figure 7). The ln CTL had the lowest amount of visceral fat, epididymal fat and peri-renal fat compared to all *fa/fa* groups. The fa 9-11 group did not show any differences in the amount of visceral, epididymal or peri-renal fat compared to fa CTL. The fa 10-12 group had the highest amount of visceral and peri-renal fat compared to all other groups but for epididymal fat, fa 10-12 had higher levels compared to fa CTL and ln CTL but not fa Base and fa 9-11.

In the Noto et al. (2007a) study, six week old *fa/fa* Zucker rats fed a 1.5% CLA mixture for 8 weeks had greater relative epididymal, peri-renal, and visceral adipose tissue weights (g/100 g bwt) than faCTL rats. Dietary CLA did not alter the adipose tissue in the lean Zucker rats. Also in our laboratory, Zirk (2005) fed 6-week old *fa/fa* Zucker rats diets containing 0.4% c9,t11 CLA or 0.4% t10,c12 CLA for 8 weeks. The *fa/fa* rats fed the t10,c12 CLA isomer had elevated epididymal, peri-renal and visceral adipose tissue (expressed relative to body weight) compared to the c9,t11 and control

groups, even though there were no differences in body weight among these groups. This supports the conclusion that t10,c12 CLA is the active isomer affecting adipose tissue.

Ryder et al. (2001) fed 7 week old ZDF rats with 1.5% CLA either as a CLA-rich butter (primarily c9,t11) or as a 50:50 synthetic CLA mixture (containing both c9,t11 and t10,c12 CLA) for 14 days. The results showed that the 50:50 group had smaller epididymal fat pads (mg/g body wt) compared to ZDF control, c9,t11 and pair-fed animals.

Nagao et al. (2003b) fed 7 weeks old Otsuka Long Evans Tokushima Fatty (OLETF) rats with 0.5% cis9,t11 CLA or 0.5% t10,cis 12 CLA for 3 weeks. The OLETF rat is model of DM-2, and develops hyperglyceridemia and glucose intolerance with hyperinsulinemia in adulthood. The OLETF rats fed t10,cis12 CLA had significantly lower peri-renal and edipidymal adipose tissue weight (g/100 g bwt) compared with control or 9-11 CLA groups. In second experiment, Nagao et al. (2003b) fed male OLETF rats a 1% mixture CLA for 4 weeks. CLA feeding decreased the wet weight of visceral adipose tissue by 23%. In a second 10 day experiment they found the t10,c12 CLA isomer promoted energy metabolism (increased oxygen consumption and increased energy expenditure) compared to the c9,t11 group, but they did not report adipose mass.

There were no significant changes in epididymal or peri- renal adipose tissue (absolute weight) in the Purushotham et al. (2006) study when Wistar rats were fed a high fat diet (20% fat by weight) for 4 weeks followed by low fat (6.5% fat by weight) control or CLA mixture diet for an additional four weeks. Wistar rats were used in this study because the authors considered them to be resistant to the adipose-lowering effects of CLA.

The above studies show that the effect of CLA on the adipose tissue depends on the rat model and the isomer. CLA as a mixture or as the t10,c12 isomer increased adipose mass in *fa/fa* Zucker rats while it decreased adipose mass in OLETF rats and ZDF rats and it had no effect in the study with Wistar rats. In these studies, it appears that the t10,c12 isomer is responsible for the changes in adipose mass. Although both the *fa/fa* and ZDF rats have a mutation in the leptin receptor, the ZDF study was only 14 days long compared to 8 weeks for the present study with *fa/fa* Zucker rats. Perhaps effects of CLA on reducing fat mass are not sustained over longer periods of time.

Liver Weight and Hepatic Steatosis

The present study showed that there were no significant differences in absolute liver weight among the *fa/fa* groups (Figure 5a). When liver weight was expressed relative to body weight, the fa Base had a greater liver weight than the fa CTL and fa CLA groups (Figure 5b). The *fa/fa* Zucker rats had a higher liver weight and liver weight/body weight ratio compared to lean Zucker rats. CLA treatment did not affect liver weight or the liver/body weight ratio. In this same study, the amount of liver lipid was measured to determine the amount of hepatic steatosis. The 10-12 CLA group had significantly lower liver lipid (%) compared to fa Base, fa 9-11 and fa CTL, and the liver lipid in the fa 10-12 was similar to the ln CTL group (Taylor and Zahradka, unpublished). Thus, this is the first study to show that t10,c12 CLA can be used to treat hepatic steatosis in older Zucker rats with established hepatic steatosis and metabolic syndrome. Interestingly, the liver lipid concentration was significantly decreased but liver weight was unchanged.

In another study by our laboratory, Noto et al. (2006) fed younger *fa/fa* rats (7 weeks old) a 1.5% CLA mixture for 8 weeks. The fa CTL rats had greater liver weight, relative liver weight and liver lipid content compared with lean animals. The CLA-fed *fa/fa* rats had reduced (37%) liver weight relative to body weight and the liver lipid content was reduced by 62% compared to fa CTL group. In fact, the relative liver weight of the CLA-fed group was similar to lean animals. Liver weight and liver lipid were the same in lean animals fed CLA or control diet. Two other studies in our lab have shown that the t10,c12 CLA isomer, but not the c9,t11 CLA isomer, is responsible for reducing the liver weight (absolute and relative liver weight), and hepatic steatosis in younger *fa/fa* Zucker rats (Stringer, 2006; Taylor and Zahradka, unpublished data).

Nagao et al. (2005) fed 6 weeks old *fa/fa* Zucker rats a 1% mixture of CLA isomers for 8 weeks. At the end of the study, the control group developed severe NAFLD. The relative liver weight was 26% less in the CLA group and this was associated with 78% reduction in the TAG accumulation in the liver. The results of this study confirmed that CLA protects *fa/fa* Zucker rats from the development of NAFLD.

In another study, Nagao et al. (2003b) fed male OLETF rats a 1% mixture of CLA for 4 weeks. Feeding the CLA diet lowered the hepatic TAG concentration, however, the authors did not report liver weight. They also stated that the OLETF rats did not develop fatty liver but did not provide any data to support this. In a second 10 day experiment, they found significant reductions of hepatic TAG concentrations in the t10,c12 CLA group as compared with the c9,t11 CLA group. Thus, it appears that the effects of t10,c12 CLA on hepatic lipid happen quickly (within days).

When Purushotham et al. (2006) fed Wistar rats with a high fat diet (20% fat by weight) for 4 weeks followed by low fat (6.5% fat by weight) control or CLA mixture diet for an additional four weeks, the results showed that CLA significantly decreased TAG accumulation in the liver by approximately 20% compared to control diet but there was no difference in liver weight.

In the current study, the results show that a t10,c12 CLA diet can increase peri-renal and epididymal adipose mass in *fa/fa* Zuckers rats without changes in body weight, and at the same time reduce hepatic steatosis without affecting liver weight. Thus, the reduction hepatic steatosis did not improve overall adiposity. CLA can alter the metabolism of different tissues. For example, in the present study, CLA altered liver lipid metabolism, but this did not lead to a positive change in body weight, epididymal or peri-renal fat. It appears that the adipose tissue is accumulating more TAGs, while liver is accumulating less. The liver can synthesize TAGs and then package them into VLDLs for transport to tissues. The lipoprotein lipase on the blood vessel wall can release the fatty acids for uptake into tissues such as adipose tissue. In terms of the present study, there could be less TAG in the liver if there is more beta-oxidation, less synthesis and/or more transport out of the liver with VLDLs. There could be more TAGs accumulating in adipose mass if they are less accessible for beta-oxidation or if there is greater uptake of fatty acids from VLDLs and chylomicrons to re-synthesize TAGs in adipose tissue. Given that body weight is not different among the *fa/fa* groups, the t10,c12 CLA is altering the distribution of lipids or lipid metabolism in liver and adipose tissue.

In summary, the studies with rat models show that CLA, and in particular the t10,c12 CLA isomer, reduces hepatic steatosis. The effects on liver weight are more

variable or not reported. The molecular mechanisms involved in the development of fatty liver are not well known yet. It has been suggested that fatty liver could be a consequence of increased lipogenesis in the liver to compensate for the reduction of fat deposition in adipose tissue (Wang, 2004). CLA isomers alter lipid liver metabolism, and as was mentioned earlier, this alteration could be the result of changes in lipid oxidation or lipid storage. In reviewing the literature, t10, c12 CLA typically has greater potency compared to other isomers (Ferramosca, 2006) and this is supported by the current study.

Effect of CLA on Lipid Droplet Number and Lipid Droplet Area

The ln CTL group had the lowest number of lipid droplets and the smallest lipid droplet areas (Figure 10 & 11). This would be expected given that lean rats had a low level of lipid in the liver when the lipid was extracted and quantified (Taylor and Zahradka, unpublished). Although the number of lipid droplets in the liver of the *fa/fa* groups was similar, the area of lipid droplets was more than five-fold larger in the fa CTL group compared to other fa groups. Given the same number of lipid droplets in these groups, the only way for lipid droplet area to go up would be for each droplet to be considerably larger in size for the faCTL group. Treatment with CLA 9-11 and CLA 10-12 both resulted in a reduction in lipid droplet area compared to faCTL. The size of the lipid droplets in these two CLA groups was similar to fa Base which indicated that the CLA isomers prevented any further increase in lipid droplet size compared to the beginning of the study. We can conclude that both 9-11 and 10-12 isomers have a positive effect on lipid droplet size in liver cells in *fa/fa* rats. However, only the 10-12

CLA group showed a reduction in hepatic steatosis when total liver lipid was measured (Taylor and Zahradka, unpublished).

This is one of the first studies to quantify the number and size of lipid droplets in liver tissue using H&E stained liver sections and software for the quantification. When the slides were being examined, it was noted that the shape of the lipid droplets was different in the two CLA groups. The fa CTL group had lipid droplets that were more oval in shape than the 9-11 group. However, these potential differences in shape were not captured with the software.

The only other study to investigate effects of CLA isomers on lipid droplet number and size was completed in our laboratory by Stringer et al. (2009). The current study and the study by Stringer et al. (2009) both investigated CLA isomers for 8 weeks duration in *fa/fa* Zucker rats, but the ages of the rats were different (6 weeks old versus 17 weeks old) at the beginning of the intervention. Stringer et al. (2009) found that the liver lipid content was reduced in *fa/fa* rats fed t10,c12 CLA but not c9,t11 CLA compared to the *fa/fa* CTL group. Although the *fa/fa* rats fed c9,t11 CLA had fewer lipid droplets compared the *fa/fa* t10,c12 group, the mean area and volume of lipid in the lipid droplets was greater in c9,t11 group and this would explain the greater liver lipid content in the c9,t11 group. The t10,c12 CLA group had more lipid droplets than the *fa/fa* CTL but the mean area and volume of lipid in the lipid droplets was less in the t10,c12 group, and thus the overall amount of hepatic lipid was less. They used the mean area and number of lipid drops in a given area to calculate the volume of lipid in lipid droplets given that lipid droplets are spherical in shape. In the present study, fa CTL and fa 9-11 had the same number of lipid droplets and the same amount of liver lipid, however, the average size of

the lipid droplets was reduced in the fa 9-11 group. Perhaps this discrepancy could be explained if the volumes for the lipid droplets were taken into account.

The Effect of CLA on Lipid Droplet Proteins

Treatment with CLA significantly decreased adipophilin protein levels in the liver of the fa 10-12 group compared to fa CTL, fa 9-11 and fa Base (Figure 12). The fa 10-12 group had hepatic adipophilin protein levels that were similar to the ln CTL, suggesting that this change is in the positive direction. Protein levels of perilipin in the liver were lower in fa CTL, fa 10-12 and fa 9-11 compared to ln CTL, while the fa Base had an intermediate level that was not different from the other groups (Figure 13). Thus, it appears that obesity reduces perilipin, however, CLA was not able to counteract this effect. By considering the fact that some drugs such as rosiglitazone increase perilipin levels (Bickel et al., 2009), we can conclude that having a higher level of perilipin is healthier than having a low level of perilipin.

Efficient fatty acid esterification into TAG and an ability to package newly synthesized TAG are necessary for viability of cells. For this reason the majority of TAG in mammalian cells is stored in droplets, which are coated with one or more proteins belonging to the PAT family (Wolins et al., 2006). Each protein associated with the lipid droplets has a unique role in TAG management. Some of these proteins drive TAG storage, whereas others regulate TAG hydrolysis. Adipophilin and perilipin are located between the cytosol and the surface of lipid droplets, and thus are proposed to regulate the lipid pool (Bickel et al., 2009). The present study shows that both perlipin and adipophilin are present in liver tissue. The effects of adipophilin in hepatic lipid metabolism have

68

been studied using adipophilin deficient mice. Adipophilin deficient mice fed high fat diets have reduced liver TAG content and liver lipid droplets that were reduced in size and number, consistent with a role of adipophilin in the formation of lipid droplets (Chang et al. 2006). The adipophilin deficient mice had normal levels of adipose tissue despite reduced hepatic steatosis. In the present study, the fa 10-12 group had slightly elevated visceral fat even though they had less hepatic steatosis than the fa CTL group.

In the fed state, perilipin protects TAG from hydrolysis in adipocytes while it promotes hormone-stimulated lipolysis in fasted animals (Brasaemle et al., 2004). In non-adipocytes, adipophilin encloses the TAG and protects it from hydrolysis. Thus, adipophilin may play the same role as perilipin as a barrier against TAG hydrolysis and this is supported by studies with knockout mice. Mice lacking perilipin do not have a large TAG storage in adipose tissue (Londos et al. 2005) and adipophilin null mice are less effective at TAG storage in liver (Wolins et al. 2006). Furthermore, overexpression of adipophilin or perilipin increases interacellular TAG storage in cultured cells. In the present study, the fa 10-12 and ln CTL rats had the lowest level of hepatic adipophilin and the lowest amount of hepatic lipid (TAG). This reduction in adipophilin levels in the fa 10-12 group could increase the amount of TAG being accessible and available to hydrolysis and thus explain the reduction in hepatic steatosis in this group. Considering that the ln CTL is a healthy animal, the results indicate that t10,c12 CLA is able to normalize both adipophilin and hepatic lipid levels. However, the effects of t10,c12 CLA on liver lipid and lipid droplet proteins appear to be mediated through adipophilin and not perilipin as perilipin was unchanged by either CLA isomer.

Lipid droplets are spherical in shape and incorporate structural proteins on their surface. Adipophilin and perilipin are important structural proteins found on the surface of lipid droplets and therefore the levels of these proteins correspond to the cumulative surface area of lipid droplets. Because these two proteins manage the packing of lipid droplets, the level of these proteins could affect the size of lipid droplets. The ln CTL group had the smallest lipid droplet size and number as well as a low level of adipophilin and a high level of perilipin compared to fa CTL. However, both 9-10 and 10-12 fa groups had a reduction in the size of lipid droplets, but no change in number of lipid droplets, compared to fa CTL, and only the fa 10-12 group showed a reduction in adipophilin protein. Thus, the reduction in hepatic steatosis and the smaller lipid droplets in the liver of fa 10-12 group were paralleled with lower adipophilin levels. However, the smaller size of lipid droplets in the fa 9-11 group must be affected by other proteins not measured in this study. As well, the smaller lipid droplets in the fa 9-11 group did not affect overall liver lipid concentration as they had the same level of hepatic steatosis as the fa CTL group. It is unclear how the fa 9-11 rats could have the same number of small lipid droplets as the fa 10-12 group yet not have a reduction in the overall liver lipid concentration. Perhaps this is a limitation of the software used to quantify the lipid droplet size and number, and the difficulty in distinguishing lipid droplets. In the future, specific stains for lipids (e.g. Oil Red O) could be used versus the H&E staining used in this project.

When Stringer et al. (2009) investigated lipid droplet proteins in the younger *fa/fa* rats fed CLA isomers for 8 weeks, they also found that the *fa/fa* t10,c12 group had lower levels of adipophilin and less total liver lipid content compared to other *fa/fa* groups.

They also found that hepatic perilipin was the same in all groups. Other members of the lipid droplet protein family, including TIP47, OXPAT, caveolin-1, as well as phospho-perilipin were not changed. Thus, the present study and the Stringer et al. (2009) study are in agreement that t10,c12 CLA reduces hepatic steatosis in *fa/fa* rats, and this is associated with a reduction in adipophilin but not perilipin in liver tissue. Results from several cell culture studies support the notion that overexpression of adipophilin is associated with increasing of lipid droplet pools and increased cellular TAG accumulation (Bickel et al., 2009).

There are very few studies in the literature which have examined lipid droplet proteins in the context of obesity and diabetes. Several drugs, including the thiazolidinediones (PPARγ agonist), have been shown to affect perilipin gene expression (Bickel et al., 2009). The expression of the perilipin gene is regulated by PPARγ. Perilipin has 3 isoforms (A, B and C). Perilipin A is the most abundant isoform and is a gatekeeper that protects lipid droplets from lipase enzymes. Under basal conditions, perilipin reduces TAG hydrolysis. In contrast when the body needs energy, perilipin A is phosphorylated and this facilitates lipase action (Bickel et al., 2009). Kim et al. (2006) conducted a study to investigate the effect of rosiglitazone (PPARγ agonist) on fat regulation and perilipin expression in 18 week old male OLETF rats. The rats were divided into 4 diet groups and provided with high fat or standard diet with or without rosiglitazone for 6 weeks. The rats fed high fat diet and treated with rosiglitazone had a lower liver TAG content but higher subcutaneous fat adjusted for body weight and a higher ratio of subcutaneous fat weight to epidydimal fat. Many studies have shown that the improvements in insulin sensitivity with TZDs are due to a redistribution of TAG

from liver and muscle to adipose tissue. In addition, TZDs increase subcutaneous adiposity without increasing visceral fat. The study by Kim et al. (2006) showed that the rosiglitazone treated group had higher perilipin expression in subcutaneous fat compared to standard diet group and that there were no differences in perilipin levels in epidydimal fat. They did not investigate perilipin in liver. Kim et al. (2006) proposed that the elevated perilipin levels were promoting more TAG accumulation in subcutaneous adipose tissue by perilipin blocking TAG lipolysis. In the present study, we do not know if CLA increased subcutaneous adipose tissue in the *fa/fa* rats or if adipose levels of perilipin were altered. Given that some effects of CLA may be mediated through PPARs and that perilipin expression is regulated by PPARγ, it is possible that CLA could affect perilipin expression through PPARγ which is found primarily in adipose tissue. The present study examined the liver where PPARα is more abundant.

Cell Culture Study

H4IIE cells treated with the PPARγ and PPARα agonists (rosiglitazone and WY14643) had less lipid accumulation by visual inspection (Figure 14) and adipophilin protein levels (Figure 15) compared to cells treated with the LXR and FXR agonists. H4IIE cells treated with OA alone or the PPARδ agonist had intermediate levels of lipid accumulation and adipophilin protein amounts that were not significantly different from either the null treated control cells or the cells treated with the LXR or FXR agonists. Although both adipophilin and perilipin could be measured in the liver tissue, this was not the case for the H4IIE cells. The perilipin antibody used to conduct Western blot analysis

of perilipin levels was inadequate for the task. The Western blotting was conducted five times with three different perilipin antibodies and yet the perilipin band was not clear.

Adipophilin and perilipin play a role in protecting lipid droplets against lipolysis by lipase enzymes in the cytosol (Bickel et al., 2009). In both the H4IIE cells and the liver tissue from the animal study, reduced levels of adipophilin were associated with less lipid accumulation. This supports a role for adipophilin in protecting TAG in lipid droplets from hydrolysis. If there is less adipophilin there would be less protection of TAG from hydrolysis and thus less lipid accumulation.

Since CLA activates PPARs and both PPARγ and PPARα agonists reduced lipid accumulation and adipophilin protein levels in the H4IIE cells, it is possible that CLA is acting through a similar mechanism *in vivo*. The liver tissue has predominantly PPARα, thus it is proposed that CLA could be acting via this transcription factor to induce the changes in liver TAG stores and adipophilin protein levels. It is also possible that CLA is acting via PPARγ and affecting perilipin in adipose tissue, but this study did not investigate lipid droplet proteins in adipose tissue.

In vitro studies show that PPARs play an important role in modulating lipid metabolism (Bickel., 2009). The present study showed that stimulating PPARs with agonists for PPARγ and PPARα can change lipid levels within cells. This is the first study to demonstrate that PPAR agonists, and specifically rosiglitazone and Wy14643, can alter levels of adipophilin in a hepatic cell culture model. Kim et al. (2006) have previously shown that rosiglitazone can increase levels of perilipin in subcutaneous adipose tissue of OLEFT rats. PPARγ activation by thiazolidinediones results in increased fatty acid uptake by adipocytes, thereby preventing lipotoxicity in liver.

Wy14643 and rosiglitazone increase lipolysis (George, 2008) and perhaps lipid droplet proteins such as adipophilin and perilipin are involved in this process as their levels determine whether or not TAGs are protected from lipolysis.

In conclusion, the anti-steatotic effects of t10,c12 CLA in older *fa/fa* Zucker rats with established obesity and metabolic syndrome were associated with reduced levels of the lipid droplet protein adipophilin in liver tissue as well as smaller lipid droplet area whereas hepatic perilipin was unchanged. Previously our laboratory has demonstrated that supplementation with t10,c12 CLA for 8 weeks decreases hepatic adipophilin but does not change perilipin in six-week old growing male *fa/fa* Zucker rats (Stringer et al., 2009). The cell culture study indicated that reductions in lipid accumulation and adipophilin levels in H4IIE cells are mediated by PPARγ and PPARα, but not LXR or FXR.

CONCLUSIONS

The major research findings can be summarized as follows:

Animal Study

- The total feed intake was higher in *fa/fa* Zucker rats compared to ln CTL rats. There were no significant differences between CLA dietary groups and fa CTL for total feed intake.

- There was no effect of CLA supplementation on weekly or final body weight.

- The fa 10-12 group had the highest amount of visceral and peri-renal fat compared to all other groups but for epididymal fat, fa 10-12 had higher levels compared to fa CTL and ln CTL but not fa Base and fa 9-11.

- The t10,c12 CLA diet increased peri-renal and epididymal adipose mass in *fa/fa* Zuckers rats without changes in body weight, and at the same time reduced hepatic steatosis without affecting liver weight. Thus, the reduction in liver weight did not improve overall adiposity.

- Treatment with CLA 9-11 and CLA 10-12 both resulted in a reduction in lipid droplet area compared to fa CTL.

- The fa CTL and fa 9-11 had the same number of lipid droplets and the same amount of liver lipid, however, the average size of the lipid droplets was reduced in the fa 9-11 group.

- The present study shows that both perlipin and adipophilin are present in liver tissue

- Treatment with CLA significantly decreased adipophilin protein levels in the liver of the fa 10-12 group compared to fa CTL, fa 9-11 and fa Base

- Protein levels of perilipin in the liver were lower in fa CTL, fa 10-12 and fa 9-11 compared to ln CTL.

Cell Culture Study

- H4IIE cells treated with the PPARγ and PPARα agonists (rosiglitazone and WY14643) had less of lipid accumulation and adipophilin protein levels compared to cells treated with the PPARβ, LXR and FXR agonists.

In conclusion, the anti-steatotic effects of t10,c12 CLA in older *fa/fa* Zucker rats with established obesity and metabolic syndrome were associated with reduced levels of the lipid droplet protein adipophilin in liver tissue as well as smaller lipid droplet area whereas hepatic perilipin was unchanged. The role of lipid droplet proteins and lipid droplets in hepatic lipid metabolism and NAFLD is a new area of research. More studies are needed in this field to provide clear insights into the biology of lipid droplets and establish their place as key organelles in human health and disease processes.

STRENGTHS AND LIMITATIONS

Strengths

- To my knowledge, this is the first study to show the effect of CLA isomers on lipid droplet proteins in a model of hepatic steatosis using the adult Zucker rat with established disease.

- This is one of the first studies to quantify the number and size of lipid droplets in liver tissue using H&E stained liver sections and image analysis software for quantification.

- The 8 weeks duration of this research is considered long term compared to other studies.

- Having 10 animals in each dietary group was adequate.

- Treating the animals with purified CLA isomers gave us the clear picture of the CLA effect on NAFLD compared to other studies with mixtures of CLA isomers.

- Using an *in vitro* model to investigate a potential mechanism for the results from animal study.

Limitations

- We studied male *fa/fa* and lean Zucker rats that are a genetic model for obesity (mutation in leptin receptor). Hepatic steatosis in humans is not due to a single gene mutation.

- The form of CLA isomers we used were free fatty acid form, while in nature and foods, CLA is found in TAG form.

- The perilipin antibody did not work for Western blotting for H4IIE cells, or it is possible that H4IIE cells do not have perilipin.

- Composition of the basal diet may influence to the results (e.g. egg white, soy or casein based diets).

- The computer software had some limitations and this could influence the histological data.

FUTURE DIRECTIONS

More research must be developed for a clear picture of the CLA effects in animals. Also more clinical research in humans is needed.

- The amount of CLA we used in this study (on a per kilogram) is much higher compared to the human diet or in supplementation studies. More work is needed to determine whether a threshold exists for CLA in the human diet.

- The mechanism by which CLA regulates lipid droplet formation requires more research.

- Experiments with mutants of H4IIE cells could be used to further investigate the role of PPARs.

- Different treatments protocols such as cell stage and time are needed.

- Specific stains for lipids (e.g. Oil Red O) could be used versus the H&E staining

REFERENCES

Adida, A., & Spener, F. (2002). Intracellular lipid binding proteins and nuclear receptors involved in branched-chain fatty acid signaling. *Prostaglandins, Leukotrienes, and Essential Fatty Acids, 67*(2-3), 91-98.

Angulo, P. (2005). Nonalcoholic fatty liver disease. *Revista De Gastroenterologia De Mexico, 70 Suppl 3*, 52-56.

Bajaj, M., Suraamornkul, S., Piper, P., Hardies, L. J., Glass, L., Cersosimo, E., et al. (2004). Decreased plasma adiponectin concentrations are closely related to hepatic fat content and hepatic insulin resistance in pioglitazone-treated type 2 diabetic patients. *The Journal of Clinical Endocrinology and Metabolism, 89*(1), 200-206.

Bass, J. Y., Caldwell, R. D., Caravella, J. A., Chen, L., Creech, K. L., Deaton, D. N., et al. (2009). Substituted isoxazole analogs of farnesoid X receptor (FXR) agonist GW4064. *Bioorganic & Medicinal Chemistry Letters, 19*(11), 2969-2973.

Bickel, P. E., Tansey, J. T., & Welte, M. A. (2009). PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. *Biochimica Et Biophysica Acta, 1791*(6), 419-440.

Bility, M. T., Devlin-Durante, M. K., Blazanin, N., Glick, A. B., Ward, J. M., Kang, B. H., et al. (2008). Ligand activation of peroxisome proliferator-activated receptor beta/delta (PPAR beta/delta) inhibits chemically induced skin tumorigenesis. *Carcinogenesis, 29*(12), 2406-2414.

Brasaemle, D. L., Dolios, G., Shapiro, L., & Wang, R. (2004). Proteomic analysis of

    proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1

    adipocytes. *The Journal of Biological Chemistry, 279*(45), 46835-46842.

Cariou, B., van Harmelen, K., Duran-Sandoval, D., van Dijk, T. H., Grefhorst, A.,

    Abdelkarim, M., et al. (2006). The farnesoid X receptor modulates adiposity and

    peripheral insulin sensitivity in mice. *The Journal of Biological Chemistry, 281*(16),

    11039-11049.

Chang, B. H., Li, L., Paul, A., Taniguchi, S., Nannegari, V., Heird, W. C., et al. (2006).

    Protection against fatty liver but normal adipogenesis in mice lacking adipose

    differentiation-related protein. *Molecular and Cellular Biology, 26*(3), 1063-1076.

Chinetti-Gbaguidi, G., & Staels, B. (2009). Lipid ligand-activated transcription factors

    regulating lipid storage and release in human macrophages. *Biochimica Et*

    Biophysica Acta, 1791(6):486-93

Choi, Y., Kim, Y. C., Han, Y. B., Park, Y., Pariza, M. W., & Ntambi, J. M. (2000). The

    trans-10,cis-12 isomer of conjugated linoleic acid downregulates stearoyl-CoA

    desaturase 1 gene expression in 3T3-L1 adipocytes. *The Journal of Nutrition, 130*(8),

    1920-1924.

Evans, M., Brown, J., & McIntosh, M. (2002). Isomer-specific effects of conjugated

    linoleic acid (CLA) on adiposity and lipid metabolism. *The Journal of Nutritional*

    Biochemistry, 13(9), 508-514.

Evans, M. J., & Kovacs, C. J. (1977). Properties of the H-4-II-E tumor cell system. I.

    growth and cell proliferation kinetics of an experimental hepatoma. *Cell and Tissue*

    Kinetics, 10(3), 233-243.

Feng, S., Yang, M., Zhang, Z., Wang, Z., Hong, D., Richter, H., et al. (2009).

Identification of an N-oxide pyridine GW4064 analog as a potent FXR agonist.

Bioorganic & Medicinal Chemistry Letters, 19(9), 2595-2598.

Ferramosca, A., Savy, V., Conte, L., Colombo, S., Einerhand, A. W., & Zara, V. (2006).

Conjugated linoleic acid and hepatic lipogenesis in mouse: Role of the mitochondrial

citrate carrier. *Journal of Lipid Research, 47*(9), 1994-2003.

Fishman, S., Muzumdar, R. H., Atzmon, G., Ma, X., Yang, X., Einstein, F. H., et al.

(2007). Resistance to leptin action is the major determinant of hepatic triglyceride

accumulation in vivo. *The FASEB Journal, 21*(1), 53-60.

Gaemers, I. C., & Groen, A. K. (2006). New insights in the pathogenesis of non-alcoholic

fatty liver disease. *Current Opinion in Lipidology, 17*(3), 268-273.

George, J., & Liddle, C. (2008). Nonalcoholic fatty liver disease: Pathogenesis and

potential for nuclear receptors as therapeutic targets. *Molecular Pharmaceutics, 5*(1),

49-59.

Granlund, L., Juvet, L. K., Pedersen, J. I., & Nebb, H. I. (2003). Trans10, cis12-

conjugated linoleic acid prevents triacylglycerol accumulation in adipocytes by

acting as a PPARgamma modulator. *Journal of Lipid Research, 44*(8), 1441-1452.

Health Canada (2009a, December 22). *Obesity.* Retrieved from < http://www.hc-

sc.gc.ca/hl-vs/iyh-vsv/life-vie/obes-eng.php>.

Health Canada (2009b, December 22). *Canadian Guidelines for Body Weight*

Classification in Adults. Retrieved from <http://www.hc-sc.gc.ca/fn-

an/nutrition/weights-poids/guide-ld-adult/qa-qr-pub-eng.php>.

Imamura, M., Inoguchi, T., Ikuyama, S., Taniguchi, S., Kobayashi, K., Nakashima, N., et al. (2002). ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *American Journal of Physiology.Endocrinology and Metabolism, 283*(4), E775-83.

Kaimal, R., Song, X., Yan, B., King, R., & Deng, R. (2009). Differential modulation of farnesoid X receptor signaling pathway by thiazolidinediones. *The Journal of Pharmacology and Experimental Therapeutics. 330*(1):125-34.

Kim, H. J., Jung, T. W., Kang, E. S., Kim, D. J., Ahn, C. W., Lee, K. W., et al. (2007). Depot-specific regulation of perilipin by rosiglitazone in a diabetic animal model. *Metabolism: Clinical and Experimental, 56*(5), 676-685.

Knox, W. E., Auerbach, V. H., & Lin, E. C. (1956). Enzymatic and metabolic adaptations in animals. *Physiological Reviews, 36*(2), 164-254.

Kraft, J., Collomb, M., Mockel, P., Sieber, R., & Jahreis, G. (2003). Differences in CLA isomer distribution of cow's milk lipids. *Lipids, 38*(6), 657-664.

Leaver, M. J., Tocher, D. R., Obach, A., Jensen, L., Henderson, R. J., Porter, A. R., et al. (2006). Effect of dietary conjugated linoleic acid (CLA) on lipid composition, metabolism and gene expression in Atlantic salmon (salmo salar) tissues. *Comparative Biochemistry and Physiology.Part A, Molecular & Integrative Physiology, 145*(2), 258-267.

Lee, K. N., Kritchevsky, D., & Pariza, M. W. (1994). Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis, 108*(1), 19-25.

Leonard, B. L., Watson, R. N., Loomes, K. M., Phillips, A. R., & Cooper, G. J. (2005). Insulin resistance in the Zucker diabetic fatty rat: A metabolic characterisation of obese and lean phenotypes. *Acta Diabetologica, 42*(4), 162-170.

Lin, H., Boylston, T. D., Chang, M. J., Luedecke, L. O., & Shultz, T. D. (1995). Survey of the conjugated linoleic acid contents of dairy products. *Journal of Dairy Science, 78*(11), 2358-2365.

Londos, C., Sztalryd, C., Tansey, J. T., & Kimmel, A. R. (2005). Role of PAT proteins in lipid metabolism. *Biochimie, 87*(1), 45-49.

Martin, G., Schoonjans, K., Lefebvre, A. M., Staels, B., & Auwerx, J. (1997). Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators. *The Journal of Biological Chemistry, 272*(45), 28210-28217.

Martinez-Fernandez, P., Hierro, L., Jara, P., & Alvarez, L. (2009). Knockdown of ATP8B1 expression leads to specific downregulation of the bile acid sensor FXR in HepG2 cells: Effect of the FXR agonist GW4064. *American Journal of Physiology.Gastrointestinal and Liver Physiology, 296*(5), G1119-29.

Miglio, G., Rosa, A. C., Rattazzi, L., Collino, M., Lombardi, G., & Fantozzi, R. (2009). PPARgamma stimulation promotes mitochondrial biogenesis and prevents glucose deprivation-induced neuronal cell loss. *Neurochemistry International, 55*(7), 496-504.

Nagao, K., Inoue, N., Wang, Y. M., & Yanagita, T. (2003a). Conjugated linoleic acid enhances plasma adiponectin level and alleviates hyperinsulinemia and hypertension in Zucker diabetic fatty (fa/fa) rats. *Biochemical and Biophysical Research Communications, 310*(2), 562-566.

Nagao, K., Wang, Y. M., Inoue, N., Han, S. Y., Buang, Y., Noda, T., et al. (2003b). The 10trans, 12cis isomer of conjugated linoleic acid promotes energy metabolism in OLETF rats. *Nutrition,* 19(7-8), 652-656

Nagao, K., Inoue, N., Wang, Y. M., Shirouchi, B., & Yanagita, T. (2005). Dietary conjugated linoleic acid alleviates nonalcoholic fatty liver disease in Zucker (*fa/fa*) rats. *The Journal of Nutrition, 135*(1), 9-13.

National Cholesterol Education Program. (2009, December 18). *Metabolic Syndrome.* Retrieved from <http:www.americanheart.org/presenter.jhtml?identifier=4756>.

Noto, A., Zahradka, P., Yurkova, N., Xie, X., Nitschmann, E., Ogborn, M., et al. (2006). Conjugated linoleic acid reduces hepatic steatosis, improves liver function, and favorably modifies lipid metabolism in obese insulin-resistant rats. *Lipids, 41*(2), 179-188.

Noto, A., Zahradka, P., Ryz, N. R., Yurkova, N., Xie, X., & Taylor, C. G. (2007a). Dietary conjugated linoleic acid preserves pancreatic function and reduces inflammatory markers in obese, insulin-resistant rats. *Metabolism: Clinical and Experimental, 56*(1), 142-151.

Noto, A., Zahradka, P., Yurkova, N., Xie, X., Truong, H., Nitschmann, E., et al. (2007b). Dietary conjugated linoleic acid decreases adipocyte size and favorably modifies adipokine status and insulin sensitivity in obese, insulin-resistant rats. *Metabolism: Clinical and Experimental, 56*(12), 1601-1611.

Papandreou, D., Rousso, I., & Mavromichalis, I. (2007). Update on non-alcoholic fatty liver disease in children. *Clinical Nutrition, 26*(4), 409-415.

Pitot, H. C., Peraino, C., Morse, P. A.,Jr, & Potter, V. R. (1964). Hepatomas in tissue culture compared with adapting liver in vivo. *National Cancer Institute Monograph, 13*, 229-245.

Public Health Agency of Canada.(2009, December 18). *The face of diabetes in Canada.* Retrieved from <http://www.phac-aspc.gc.ca/cd-mc/diabetes-diabete/face-eng.php>.

Purushotham, A., Shrode, G. E., Wendel, A. A., Liu, L. F., & Belury, M. A. (2007). Conjugated linoleic acid does not reduce body fat but decreases hepatic steatosis in adult Wistar rats. *The Journal of Nutritional Biochemistry*, 18(10), 676-84.

Reddy, J. K., & Rao, M. S. (2006). Lipid metabolism and liver inflammation. II. fatty liver disease and fatty acid oxidation. *American Journal of Physiology.Gastrointestinal and Liver Physiology, 290*(5), G852-8.

Rodriguez-Alcala, L. M., & Fontecha, J. (2007). Hot topic: Fatty acid and conjugated linoleic acid (CLA) isomer composition of commercial CLA-fortified dairy products: Evaluation after processing and storage. *Journal of Dairy Science, 90*(5), 2083-2090.

Ryder, J. W., Portocarrero, C. P., Song, X. M., Cui, L., Yu, M., Combatsiaris, T., et al. (2001). Isomer-specific antidiabetic properties of conjugated linoleic acid: Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. *Diabetes, 50*(5), 1149-1157.

Sakono, M., Miyanaga, F., Kawahara, S., Yamauchi, K., Fukuda, N., Watanabe, K., et al. (1999). Dietary conjugated linoleic acid reciprocally modifies ketogenesis and lipid secretion by the rat liver. *Lipids, 34*(9), 997-1000.

Scholz, H., Lund, T., Dahle, M. K., Collins, J. L., Korsgren, O., Wang, J. E., et al. (2009). The synthetic liver X receptor agonist GW3965 reduces tissue factor production and inflammatory responses in human islets in vitro. *Diabetologia, 52*(7), 1352-62.

Silveira, M. B., Carraro, R., Monereo, S., & Tebar, J. (2007). Conjugated linoleic acid (CLA) and obesity. *Public Health Nutrition, 10*(10A), 1181-1186.

Stringer. M. D., Zahradka. P, DeClercq. C.V., Ryz. R. N., Diakiw. R., Burr. L.L., Xie. X., et al. (2009). Improved hepatic steatosis by trans-10,cis-12 conjugated linoleic acid in obese, insulin-resistant rats is associated with smaller lipid droplets and lower levels of adipophilin. Submitted manuscript.

Sugden, M. C., & Holness, M. J. (2008). Role of nuclear receptors in the modulation of insulin secretion in lipid-induced insulin resistance. *Biochemical Society Transactions, 36*(Pt 5), 891-900.

Takaya, K., Ogawa, Y., Isse, N., Okazaki, T., Satoh, N., Masuzaki, H., et al. (1996). Molecular cloning of rat leptin receptor isoform complementary DNAs - identification of a missense mutation in Zucker fatty (*fa/fa*) rats. *Biochemical and Biophysical Research Communications, 225*(1), 75-83.

Taylor, C. G., & Zahradka, P. (2004). Dietary conjugated linoleic acid and insulin sensitivity and resistance in rodent models. *The American Journal of Clinical Nutrition, 79*(6 Suppl), 1164S-1168S.

Truett, G. E., Walker, J. A., & Harris, R. B. (2000). A developmental switch affecting growth of fatty rats. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 279*(6), R1956-1963.

Tsuboyama-Kasaoka, N., Takahashi, M., Tanemura, K., Kim, H. J., Tange, T., Okuyama, H., et al. (2000). Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes, 49*(9), 1534-1542.

Upton, R., Widdowson, P. S., Ishii, S., Tanaka, H., & Williams, G. (1998). Improved metabolic status and insulin sensitivity in obese fatty *(fa/fa)* Zucker rats and Zucker diabetic fatty (ZDF) rats treated with the thiazolidinedione, MCC-555. *British Journal of Pharmacology, 125*(8), 1708-1714.

Wagner, N., Jehl-Pietri, C., Lopez, P., Murdaca, J., Giordano, C., Schwartz, C., et al. (2009). Peroxisome proliferator-activated receptor-β stimulation induces rapid cardiac growth and angiogenesis via direct activation of calcineurin. *Cardiovascular Research, 83*(1), 61-71.

Wang, Y., & Jones, P. J. (2004). Dietary conjugated linoleic acid and body composition. *The American Journal of Clinical Nutrition, 79*(6 Suppl), 1153S-1158S.

Wang, Y. M., Nagao, K., Ujino, Y., Sakata, K., Higa, K., Inoue, N., et al. (2005). Short-term feeding of conjugated linoleic acid does not induce hepatic steatosis in C57BL/6J mice. *Journal of Nutritional Science and Vitaminology, 51*(6), 440-444.

Wendel, A. A., & Belury, M. A. (2006). Effects of conjugated linoleic acid and

troglitazone on lipid accumulation and composition in lean and Zucker diabetic fatty

(*fa/fa*) rats. *Lipids, 41*(3), 241-247.

Wierzbicki, M., Chabowski, A., Zendzian-Piotrowska, M., & Gorski, J. (2009).

Differential effects of in vivo PPAR alpha and gamma activation on fatty acid

transport proteins expression and lipid content in rat liver. *Journal of Physiology and*

Pharmacology : An Official Journal of the Polish Physiological Society, 60(1), 99-

106.

Wojcicka, G., Jamroz-Wisniewska, A., Horoszewicz, K., & Beltowski, J. (2007). Liver X

receptors (LXRs). Part I: Structure, function, regulation of activity, and role in lipid

metabolism. *Postepy Higieny i Medycyny Doswiadczalnej, 61*, 736-759.

Wolins, N. E., Brasaemle, D. L., & Bickel, P. E. (2006). A proposed model of fat

packaging by exchangeable lipid droplet proteins. *FEBS Letters, 580*(23), 5484-5491.

Xu, A., Wang, Y., Keshaw, H., Xu, L. Y., Lam, K. S., & Cooper, G. J. (2003). The fat-

derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver

diseases in mice. *The Journal of Clinical Investigation, 112*(1), 91-100.

Yoshikawa, T., Ide, T., Shimano, H., Yahagi, N., Amemiya-Kudo, M., Matsuzaka, T., et

al. (2003). Cross-talk between peroxisome proliferator-activated receptor (PPAR)

alpha and liver X receptor (LXR) in nutritional regulation of fatty acid metabolism. I.

PPARs suppress sterol regulatory element binding protein-1c promoter through

inhibition of LXR signaling. *Molecular Endocrinology, 17*(7), 1240-1254.

Yau, L., Elliot, T., Lalonde, C., & Zahradka, P. (1998). Repression of phosphoenolpyruvate carboxykinase gene activity by insulin is blocked by 3-aminobenzamide but not by PD128763, a selective inhibitor of poly(ADP-ribose) polymerase. *European Journal of Biochemistry/ FEBS, 253*(1), 91-100.

Younossi, Z. M., Diehl, A. M., & Ong, J. P. (2002). Nonalcoholic fatty liver disease: An agenda for clinical research. *Hepatology, 35*(4), 746-752.

Zahradka, P., Werner, J., & Yau, L. (1998). Expression and regulation of the insulin-like growth factor-1 receptor by growing and quiescent H4IIE hepatoma. *Biochimica Et Biophysica Acta, 1375*(1-2), 131-139.

Zhou, Y. M., Wen, Y. H., Kang, X. Y., Qian, H. H., Yang, J. M., & Yin, Z. F. (2008). Troglitazone, a peroxisome proliferator-activated receptor gamma ligand, induces growth inhibition and apoptosis of HepG2 human liver cancer cells. *World Journal of Gastroenterology, 14*(14), 2168-2173.

Zirk, M. M. (2005). *The effect of conjugated linoleic acid (CLA) isomers on leptin, adiponectin and peroxisome proliferator-activated receptor (PPAR) expression and their influence on procine smooth muscle cells.* MSc. Thesis, University of Manitoba.